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PERIODONTAL IMPLICATIONS OF SALVADORA PERSICA L.

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Periodontal Implications of *Salvadora Persica* L.

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This thesis is dedicated to Prophet Mohammed (Peace be upon him), who was the great inspiration to this work

“If I did not fear imposing hardship on my Ummah (followers) I would have made the use of Siwak obligatory at the time of every prayer” *Prophet Mohammad*

To my mother, who passed away 24th Aug 2018, your love will lighten my way, and your memory will forever be with me.

To my father, who gives me the support and strength.

To the one and only, Layan who means the life to me.

“Risk the fall, just to know how it feels to fly”

Dave Mirra

ABSTRACT

Periodontal disease is a major public health problem throughout the world and is the most common cause of tooth loss in adults. For periodontitis to develop, specific gram-negative, anaerobic bacteria must predominate in the subgingival microflora, but simple the presence of these bacteria is insufficient to cause periodontal disease as disease progression involves a complex, sequential relationship between infection, inflammation and tissue destruction. Treatment of periodontal disease targets inflammation through reduction of pathogenic bacteria, eliminating of pathogenic pockets, and use of mechanical debridement techniques along with chemically effective plaque-control agents. To treatment efficacy, disease management should be a holistic approach with medications that have proven antibacterial, and anti-inflammatory properties.

Popular concern about the side effects of synthetic drugs and the increasing antibiotic resistance is significant and growing. In the periodontal field, efforts to find natural antimicrobial agents for preventing and treating periodontal disease have been stepped up.

Many communities use the Miswak, a chewing stick made from the roots, twigs, or stems of *Salvadora persica* L., as an oral health tool. Studies have indicated that the Miswak has both an antibacterial and an anti-inflammatory effect, conferred by benzyl isothiocyanate (BITC), the major multi biologically active component.

Hence, the overall aim of this thesis was to investigate the possible chemical effects of chewing sticks made from *S. persica* L., Miswak, in order to create optimum-use guidelines for the Miswak as a way of improving the treatment efficacy of periodontal disease.

Study I, a double-blind, cross-over trial evaluated the efficacy of active and inactive *S. persica* L. on dental plaque, subgingival microbiota, and gingival inflammation in 24 patients with mild-to-moderate periodontitis. Clinical parameters were evaluated before and immediately after all experimental periods. Samples of subgingival plaque and gingival crevicular fluid were also taken. Plaque samples were analyzed with a DNA-DNA hybridization technique. Compared with pre-treatment values, populations of 16 bacterial species increases significantly in the group using a placebo inactive Miswak, ($p < 0.05$), while no species showed a similar change in the group using active Miswak. The plaque and gingival indices on all tooth surfaces, however, differed non-significantly between the active and inactive Miswak groups.

Study II measured the amount of BITC released into the mouth after brushing with Miswak and assessed its retention time in saliva. The study also tested the antibacterial and cytotoxic efficacy of the salivary BITC. Salivary BITC and BITC on used brushes were quantified using solid phase microextraction and gas chromatography-mass spectrometry. The antibacterial effects of BITC and Miswak Essential oil (EO) against *Aggregatibacter actinomycetemcomitans*, *Haemophilus influenzae*, and *Porphyromonas gingivalis*; and cytotoxic effects on human gingival fibroblasts and oral keratinocytes were investigated. The highest concentrations of the active compounds were detected in saliva after using a Miswak tip for one time and immediately. Concentrations were significantly decreased when the

Miswak tip was used more than once and thus after 10 minutes. Miswak EO and BITC inhibited the growth of the tested bacteria in a dose dependent manner, with *P.gingivalis* being the most sensitive. A methyl tetrazolium-based MTT assay found BITC and Miswak EO to be cytotoxic to gingival fibroblasts while oral keratinocytes exhibiting resistance, which suggests that, to ensure maximum effect, the Miswak tip should be cut before each use.

Study III investigated the anti-inflammatory activities of Miswak EO from *S. persica* and the main antimicrobial compound BITC by evaluating their effect on the secretion of pro-inflammatory mediators from human gingival fibroblasts and oral keratinocytes: interleukin (IL)-6, IL-8, and matrix metalloproteinase (MMP)-1 from IL-1 β (300 pg/ml) stimulated and non-stimulated gingival fibroblasts and oral keratinocytes. An ELISA assessment found a significant decrease in the levels of IL-1 β -induced IL-6 and IL-8 in gingival fibroblasts and oral keratinocytes treated with Miswak EO and BITC. In both cell types, levels of secreted MMP-1 were unaltered.

Viewing these results, we concluded that Miswak has a weak effect on the subgingival microbiota of patients with periodontitis. Cutting a fresh Miswak tip each brushing time is necessary in order to attain a sufficient antibacterial effect. Claimed brushing frequencies with Miswak of five times a day should be considered for maximal effect. Results also showed that Miswak has an anti-inflammatory effect on the pro-inflammatory mediators secreted by gingival fibroblasts and oral keratinocytes.

Since periodontitis is a hyper-inflammatory reaction to periodontal pathogens, a treatment that combines an anti-bacterial and an anti-inflammatory effect, like Miswak could be potentially new path of treating periodontitis in a non-synthetic, natural manner.

LIST OF SCIENTIFIC PAPERS

- I. Chemical effects of *Salvadora persica* chewing sticks made of *Salvadorapersica*.
Albertain R, Ibrahim L, Bhangra S, Rosengren A, Gustafsson A.
Int J Dent Hyg. 2018 Nov;16(4):535-540. doi: 10.1111/idh.12359.
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- II. Investigations of a possible chemical effect of *Salvadora persica* chewing sticks.
Albertain R, Azeem M, Wondimu Z, Lindberg T, Borg-Karlson AK, Gustafsson A.
Evid Based Complement Alternat Med. 2017;2017:2576548. doi: 10.1155/2017/2576548. Epub 2017 Apr 18.
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- III. The anti-inflammatory effect of *Salvadora Persica* on interleukin-1 β stimulated gingival fibroblasts and oral keratinocytes.
(Submitted)
Albertain R, Bahammam M, Boström E, Gustafsson A.

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LIST OF ABBREVIATIONS

<i>S. persica</i>	<i>Salvadora persica</i>
BA	benzaldehyde
BC	benzyl cyanide
BITC	benzyl isothiocyanate
BoP	bleeding on probing
CHX	Chlorhexidine
DMEM	Dulbeco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
EO	essential oil
GC-MS	gas chromatography-mass spectrometry
GI	gingival index
hGFs	human gingival fibroblasts
HS	head space
IL	Interleukin
ITCs	isothiocyanates
MMP	matrix-metalloproteinase enzyme
MTT	methyl tetrazolium
OK	oral keratinocytes
PBS	phosphate-buffered saline
PI	plaque index
SPME	solid-phase micro extraction
VPI	visual plaque index

1 INTRODUCTION

Literature review

Periodontal disease is recognized as a major public health problem throughout the world and is the most common cause of tooth loss in adults. “*Periodontal disease*” is a general term used to describe inflammatory conditions that include the gingiva, the periodontal ligament and the alveolar bone. Colonization and overgrowth of certain bacterial species, with a dominance of Gram-negative anaerobic bacteria subgingivally, represent the principle etiological factor for initiation and progression of inflammation and tissue destruction in a susceptible host (1). For periodontitis to develop, specific Gram-negative, anaerobic bacteria must predominate in the subgingival microflora, but simply the presence of these bacteria is insufficient to periodontal disease: disease progression involves a complex, sequential relationship between infection, inflammation, and tissue destruction (2).

Prevention of periodontitis is clearly related to preventing the formation of/or eradicating the microbial biofilm; prevention of gingivitis is considered to be the prime prophylactic measure for chronic periodontitis (3). Growth in our knowledge and understanding of the mechanisms by which pathogenic bacteria induce the inflammatory response in initiation and progression of periodontal disease. Initially, an inflammatory response, thus initiating or furthering the progression of periodontal disease has been rapid. Initially the inflammatory response is functional (gingivitis) and controls the infection, but an overly strong response can lead to extensive tissue degradation, that is periodontitis. If chronic inflammation can be controlled (by reducing the bacterial load) then the tissues may potentially be driven towards repair rather than destruction.

Periodontal treatment aims to decrease inflammation by reducing the number of pathogenic bacteria and eliminating pathogenic pockets. Conventional therapy focusses mainly on mechanical removal of calculus and the subgingival biofilm. Mechanical infection controls, however, are often insufficient, and there is a need for adjunctive treatments such as chemical plaque control and antibiotics.

To enhance treatment efficacy, disease management should be conducted in a holistic approach with medications that have scientifically proven antibacterial, and anti-inflammatory properties. Popular concern about the side effects of synthetic drugs and an increasing antibiotic resistance is significant and growing. In the periodontal field, efforts to find natural antimicrobial agents for preventing and treating of periodontal disease have been stepped up. Pathogenic resistance to conventional oral therapy drugs is also raising public awareness of natural alternatives for oral health (4). In the wake of these shortcomings, plant and other natural antibacterial substances are attracting attention as possible antimicrobials that could be incorporated into mouth rinses and toothpastes (4).

Active chemical ingredients have been isolated from natural products like plants, fungus and bacterial species. It is estimated that plant material is present in, or has provided the models for, 50% of Western drugs (5). Many commercially proven drugs used in modern medicine

were initially used in crude form in traditional healing practices, suggesting potentially useful biological activity. Since many natural active chemical ingredients are included in drugs and other oral health products, use of their raw source plant materials can be justifiable, as is the case with *S. persica* chewing sticks, which the WHO recommends (6).

The primary benefits of plant-derived medicines are that they are relatively safer than synthetic alternatives and offer profound therapeutic benefits and more affordable treatment (7). The use of plant extracts, as well as other alternative forms of medical treatments, begin to have a great popularity in the late 1990s (8). Evidence from many recent studies confirm that essential oils (EOs) may be suitable additives in products maintaining oral hygiene or preventing dental disease (9). EOs have been used in traditional medicine since the dawn of recorded history.

Around the world, researches are directing their efforts toward characterizing the biological effects of EOs which include antimicrobial, antiviral, anti-mutagenic, antioxidant, anticancer, and anti-inflammatory activities. EOs are complex mixture of compounds that possess low molecular weight (usually less than 500 Daltons) and can be extracted by steam distillation, hydro-distillation or solvent extraction. One or two of the major components of a EO usually determine its bioactivities (10) Takarade et al. 2004 (11) tested several EOs and found that Manuka oil and tea tree oil had a strong antibacterial activity against periodontopathic and cariogenic bacteria. Sofrata et al. 2011 (12) analyzed EO from the roots of *S. persica* L. extracted by steam distillation, and found that benzyl isothiocyanate (BITC), the major active component, had potentially efficacy as an antimicrobial substance with strong activity against many Gram-negative pathogens. A group of scientist in Saudi Arabia found that *S. persica* had high antioxidant activities (13). A recent study by Ocheng et al. (14) analyzed EOs from 10 Ugandan aromatic medicinal plants and showed significant growth inhibitory effects on periodontopathic bacteria like *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, moderate effects on cariogenic *Streptococcus mutans*, and the least effect on *Lactobacillus acidophilus*.

A systematic review by Stoeken et al. 2007 (15) evaluated 11 RCTs on the long term effects on dental plaque and gingivitis, of a mouthwash containing EOs. The review found that EOs provide an additional benefit concerning plaque and gingivitis reduction compared to placebo when used as an adjunct to unsupervised oral hygiene.

In 1987, the American dental association approved essential oil mouthwash (16, 17). Another mouthwash approved by the American dental association is chlorhexidine (CHX) which is currently the most effective, commercially available antiplaque agent. CHX is a cationic bisbiguanide with a broad antimicrobial effect, and has been shown effectiveness in controlling gingivitis in long-term studies (18).

However, CHX has several side effects, including altered taste perception (dysgeusia) and staining, which limit its long-term use (19). There are also concerns about deleterious effects on human gingival fibroblasts (hGFs): effects on cell morphology, viability, and function. In one study (20), hGFs appeared round and retracted from root surfaces treated with 0.2% CHX. With 2% CHX, cells had a foamy appearance and most of the cytoplasm seemed to have been

extracted from the cells. In the same study, CHX reduced cell proliferation in a dose-dependent manner. Cells treated with 0.12% CHX were reported to experience 100% inhibition of proliferation. Yet, another study reported that CHX displayed cytotoxic effects toward hGFs at concentrations lower than those used in clinical practice (21).

Many clinical trials have evidenced the antiplaque and anti-inflammatory efficacy of EO mouthrinses. At the same time concerns are being raised about the inconclusive evidence on the efficacy of adding herbal extracts to mouthrinses (22). Many promising natural compounds, however are potentially efficacious in the treatment of oral health diseases and warrant further high-quality studies.

Numerous populations around the world use various plants as chewing sticks for maintaining good oral health. The chewing stick is one of the oldest oral hygiene aids in history(23) Throughout the world, over 180 species of plants have been used as chewing sticks, Miswak is harvested from *S. persica* L., also known as the Arak tree, and is the main plant used for obtaining chewing sticks from East Africa to the Asian subcontinent, including Saudi Arabia (24, 25).

S. Persica L. is a small tree or shrub up to 30 cm in diameter with a crooked trunk, and is rarely more than 3meters in height (Fig. 1). Although twigs, and stems have been used as oral hygiene tools(26) it is far more common to use the roots. The Miswak is prepared by cutting the roots or twigs into 15 cm length sticks. The stick usually has a diameter of about 0.7 to 1 cm and is washed with water to remove the sand. People usually strip off a short length of the bark layer from one end (about 0.7 cm), then chew the end a little to separate the fibers until they become like the fibers of a normal tooth brush. The Miswak is then used to brush the teeth (25). Fresh Miswak is brown in color, with a hot, pleasant taste.

Miswak through the centuries and Derivation of the botanical name *Salvadora persica* L.

Dr Laurent Garcin, botanist, traveler, and plant collector suggested the term *Salvadora* in 1749 in honor of the Barcelonan apothecary Juan Salvadory Bosca (1598–1681); *persica* refers to Persia. And the standard author abbreviation L. is used to indicate Carl Linnaeus (1707–1778), a Swedish botanist, physician, and zoologist, the father of modern taxonomy. The toothbrush tree, *S. Persica* L., locally called *Miswak* or *Siwak*, is a member of the *Salvadoraceae* family.

Using a Miswak to brush teeth is an ancient pre-Islamic custom. The Miswak is considered to be the precursor of the modern-day toothbrush. The first recorded use of chewing sticks (the Miswak) was by the Babylonians around 3500 BC, which is where the origin of the modern toothbrush took place. Elsewhere ancient Greek and Roman literature discuss chewing on toothpicks to help clean the teeth and mouth (27).

The use of a chewing stick conforms to the notion of primary health care and has long-established associations with certain cultural and religious beliefs. The spread of Islamic culture had a significant influence on the propagation and use of Miswaks, the Prophet Muhammed

was an enthusiastic supporter of its use as a “purgative for the mouth”, and he developed rules and rituals for the correct and effective use of the Miswak. He has been quoted as saying: “If I did not fear imposing hardship on my Ummah (followers) I would have made its use obligatory at the time of every prayer”, “Chewing stick is a purification for the mouth and a means of the pleasure of the Lord”. The belief of the Prophet (peace be upon him) regarding good oral hygiene was so profound that on his death-bed, he requested his wife to prepare his Miswak (28).

One of Mohammed’s biographers wrote: “Even the approach of death did not keep the Prophet from demanding the “Siwak” because it is the most elegant thing that one can use and the most fitting to be found beautiful, for it makes the teeth white, clarifies the understanding, makes the breath fragrant, extinguishes the gall, dries up the phlegm, strengthens the gums around the teeth, makes the glance clear, sharpens the power of the vision, opens the bowels and whets the appetite”.

This testimonial suggests why Muslims have used the Miswak for hundreds of years and why, for some, it is not only a good personal hygiene but a spiritual custom.

Western travelers and explorers have described the use of chewing sticks in the Sahara region and Sudan since 1889(28). Chewing sticks are known by different names in different cultures: “Miswak” or “Siwak” in Arabic, “Koyoji” in Japanese, “Qesam” in Hebrew, “Mefaka” in Amharic, and “Mastic” in Latin(28) .



Fig. 1. Arak tree (*Salvadora persica* L.) is a shrub growing naturally in the desert.
Photo adapted from www.quora.com.

Chemical composition:

The beneficial effects of Miswak, in terms of oral hygiene and dental health, can be attributed to the mechanical action of brushing and the chemical components found in *S. persica* (29). Chemical analyses have found that Miswak contains numerous, natural constituents that are known to promote oral health.

A preliminary investigation of the components in Miswak by AlFarooqi and Sirvastava(30) found various salts, mostly chlorides; a fairly large concentration of Alkaloidal constituents benzyl isothiocyanate (BITC), and negligible amounts of tannins and saponins. They concluded that the chemical composition of the root-bark could be beneficial for brushing the teeth. Miswak also contains flavonoids, including kaempferol, quercetin, quercetin rutin, and a quercetin glucoside(31). Ezmirly and El.naser (32) demonstrated that BITC is an end-product derived from the enzymatic hydrolysis of the glucosinolate present in the plant. According to Chawla(33), chewing sticks like Neem (*Azadirachta indica*), *S. persica*, and *Accacia Arabica* contain reasonable amounts of fluoride. Several of the *S. persica* constituents mentioned above can contribute to the oral health.

The antimicrobial properties of *S. persica* root extracts are attributed mainly to BITC, the major antibacterial constituent in the EO of the *S. persica* roots (12, 34). BITC exhibits a strong and rapid bactericidal effect against the gram-negative bacteria, associated with periodontal disease while inhibiting the growth and acid production of *S.mutans*(12)The silica component of the Miswak acts as an abrasive material to remove stains and whitens teeth(30).

Miswak effects on oral health:

Miswak has been reported to have anti plaque(35) and anti caries(36, 37) effects. Epidemiological studies have reported less, dental plaque and fewer caries among Miswak users than regular brush users (38, 39). Several *in vitro* and *in vivo* studies have found that alcoholic and aqueous extracts of *S. persica* are strongly antimicrobial for many aerobic and anaerobic bacteria like *S. mutans* and *Eikenella corrodens*(40-43). *In vivo* studies have shown that use of Miswak chewing sticks, Miswak extract and *S. persica* mouth wash reduced salivary bacteria counts, improved gingival health and lowered caries rate (44-46). Some studies have reported low to moderate antimicrobial activity for ethanolic and aqueous extracts of *S. persica* (47, 48). Another found that Miswak pieces embedded in agar or suspended in the air above the agar plate had much stronger inhibitory effects than aqueous Miswak extracts (36). Another study used transmission electron microscopy to illustrate how the EO of the *S.persica* root and pure BITC affect bacteria; EO and BITC both had a dramatic effect on the cell membrane causing the membrane protrude until it lost its integrity(12).

Several clinical studies have shown that regular use of chewing sticks made of *S. persica* reduce plaque and lower gingival indices (47-49). *In-vitro* studies indicate that, of a variety of common oral bacteria, members of the genus *Streptococcus* (including the *S.mutans*) are especially sensitive to the antimicrobial activities of *S. persica* (25). Guile et al concluded in a comprehensive survey of several thousand of Saudi Arabian schoolchildren that the low

incidence of gingival inflammation was attributable to the practice of cleaning the teeth with Miswak (50). Another study showed that the risk of dental caries for each tooth in the control group was 9.35 times more than in the Miswak user group (37).

The Miswak has an immediate effect on bacteria, with *S. mutans* was more susceptible than *lactobacilli* to the antibacterial activity of the chewing stick (44). One study found that an experimental group using a Miswak mouthwash had significantly lower gingival index, plaque index, and bleeding index without any reported side effects (51), while another study has reported, an association between use of Miswak and gingival recession (26)

Many studies have demonstrated the antibacterial, antifungal, and antiviral activities of BITC and flavonoids separately (31, 52-55). In Addition interest in the bioactive compounds of *S. persica*, especially its antioxidant compounds, is growing (56). By definition, antioxidants are substances that, when present in low concentrations, markedly delay or prevent reactive oxygen species (ROS) from oxidizing important biomolecules, such as a fatty acids, DNA, or proteins)(57). Oxidative processes have been linked to a variety of diseases including cardiovascular diseases, cancers(58) neurodegenerative diseases as Alzheimer's disease(59), and inflammatory diseases(60) among others.

Natural plant antioxidants can be considered a type of preventive medicine. Some investigators have suggested that two thirds of the world's plants species have medicinal value; in particular , many medicinal plants are considered to have great antioxidant potential (61) .

Mohamed and Khan (56) reported that furan derivatives containing hydroxyl groups could possess antioxidant activities. Antioxidant enzymes, high levels of peroxidase and low level of catalase and poly-phenoloxidase have also been detected in chewing stick extracts. The synergistic actions of antioxidant compounds and antioxidant enzymes could make chewing sticks a good tool for oral health and food purposes.

Research over the past three decades has provided extensive preclinical evidence for the efficacy of various isothiocyanates (ITCs) against cancer in preclinical models. Benzyl isothiocyanate (BITC) is one such compound with the ability to inhibit chemically induced cancer, oncogenic-driven tumor formation, and human tumor xenografts in rodent cancer models. Prior work also has established that BITC has the ability to influence carcinogen metabolism and signaling pathways relevant to tumor progression and invasion (62).

AIMS

Overall aim

The general aim of this thesis was to explore mechanisms for a possible chemical effect of chewing sticks made from *Salvadora persica* L.

Specific aims

1. To evaluate the efficacy of active and inactive *S. persica* L. on dental plaque, subgingival microbiota, and gingival inflammation in patients with chronic periodontitis.
2. To formulate guidelines for optimal use of Miswak and to investigate the potential antibacterial and cytotoxic effects of released amounts of antibacterial compounds.
3. To investigate whether the essential oil extract from Miswak and the BITC compound have an inhibitory effect on the extra cellular release of IL-6, IL-8, and MMP-1, in human gingival fibroblasts (hGFs) and oral keratinocytes stimulated with IL-1 β .

2 MATERIALS AND METHODS

2.1 PLANT USED IN THIS PROJECT

All Miswak used in this project came from the roots of the *Salvadora persica* L. shrub. Miswak was harvested by a local farmer in Jizan, Saudi Arabia, which is a natural cultural area rich in natural arak trees. A professional farmer confirmed identification of the plant. The Miswak was sent on a 5 day journey from Jizan to Karolinska Institutet, Stockholm, where it was cleaned and vacuum packed upon arrival and stored at -80°C.

The EO was extracted from the *S. persica* L. roots by steam distillation microextraction (Fig. 5). The compounds in the EO were identified by gas chromatography-mass spectrometry (GC-MS) which revealed three putative antibacterial components; Benzyl isothiocyanate (BITC;CID: 2346) 74.42 %, Benzylcyanide (BC;CID: 8794) 16.26%, and benzaldehyde (BA;CID: 240) 0.67%.

As a placebo, some of the *S.persica* roots were deactivated by boiling for 2 h. Inactivity was confirmed by *in vitro* testing with the Gram-negative anaerobic bacteria *Haemeophilus influenzae* (12). Each subject received four fresh Miswaks. They were instructed to refrigerate the unused sticks until use.

2.2 PAPER I

2.2.1 Study participants

Twenty-eight subjects aged 18- 71 y (16 females and 12 males) consented to participate and were enrolled in the study. None of the participants had used Miswak previously. Participants had slight to moderate periodontitis.

The inclusion criteria were age ≥ 18 y, > 20 teeth, and at least one pathological gingival pocket with a depth of 4–8 mm. The exclusion criteria were regular use of Miswak, pregnancy or lactating, treatment with antibiotics for any medical or dental condition within 1 month prior to the commencement of the trial or use of antibiotics during the trial, gingival pockets deeper than 8 mm, and reduced motor skills. Since all patients would be using both active and inactive Miswak, exclusion criteria such as chronic diseases, smoking, and long-term use of medication were not applicable.

2.2.2 Study design

The study was a double-blind, randomized cross-over study, that is, neither the clinicians nor the participants would know who was using active or inactive Miswak in the two arms of the study. Deactivated Miswak functioned as a placebo. The participants were randomly given active or inactive Miswak for the first arm of the study and the other form of Miswak for the second arm. Each arm was 3 wk, with a wash-out period of 5 wk in-between arms. During the wash-out period, the patients resumed their usual daily oral hygiene and did not use Miswak.

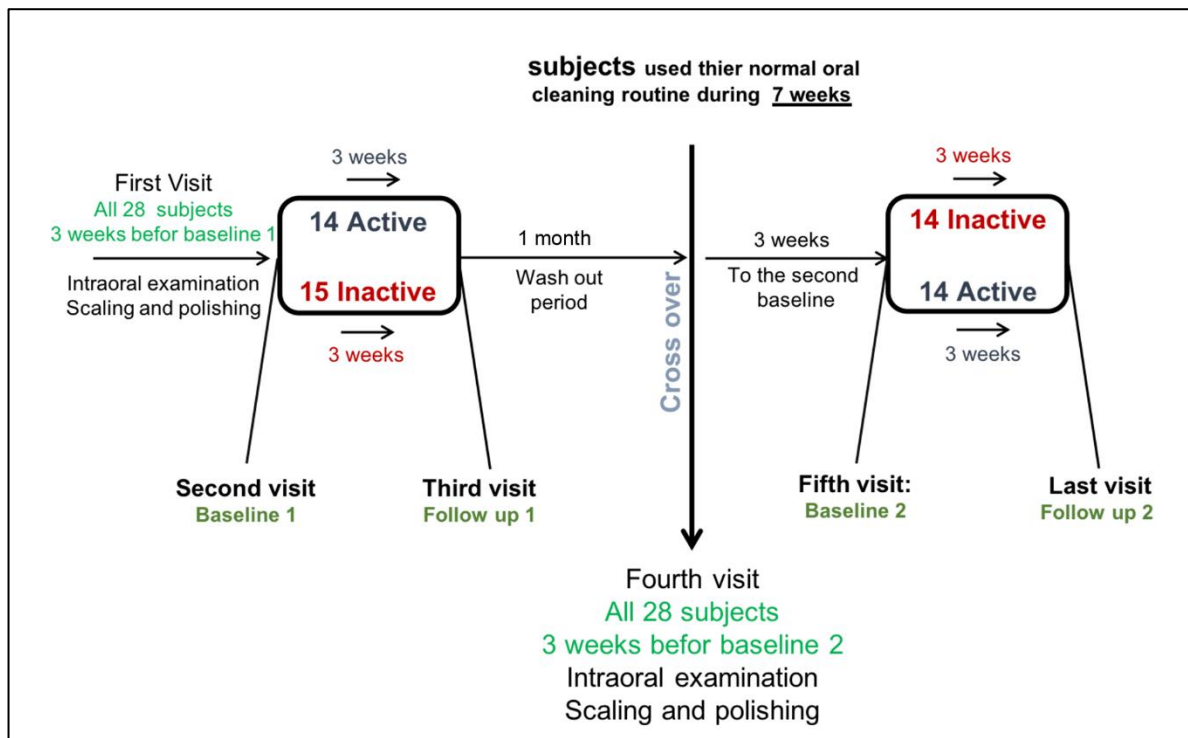


Fig. 2. Study flow chart of paper I

All subjects received six Miswak sticks that were 20 cm long and 7 mm in width. They were instructed to refrigerate the sticks until use. The study was performed according to the following plan (Fig. 2):

1st visit – Baseline minus 3 wk. The participants underwent an intraoral examination, scaling, and professional tooth cleaning. They were instructed to continue their usual oral hygiene habits until they returned 3 wk later. They were able to see and taste a Miswak.

2nd visit – Baseline. Participants were clinically examined. Clinical variables (PI, VPI, GI, BoP) were registered at four sites on each tooth (buccal, mesial, distal and lingual). The participants were instructed in the use of Miswaks with photos and given a brushing schedule (Fig. 3). All participants refrained from all other oral hygiene procedures (i.e., toothbrushing, flossing or use of interdental aids).

3rd visit – Baseline plus 3 wk. Same tests as in the 2nd visit was conducted.

5-wk washout period.

4th visit – 2nd Baseline minus 3 wk. Same treatment as in the 1st visit

5th visit – 2nd Baseline. Crossover. Same tests as in the 2nd visit. The participants were given the other form of Miswak, which they had not used in the first arm.

6th visit - 2nd Baseline plus 3 wk. Same tests as in the 2nd visit

The study was ethically approved from the regional Research Ethics Committee in Stockholm (# 2012/609-31/1).

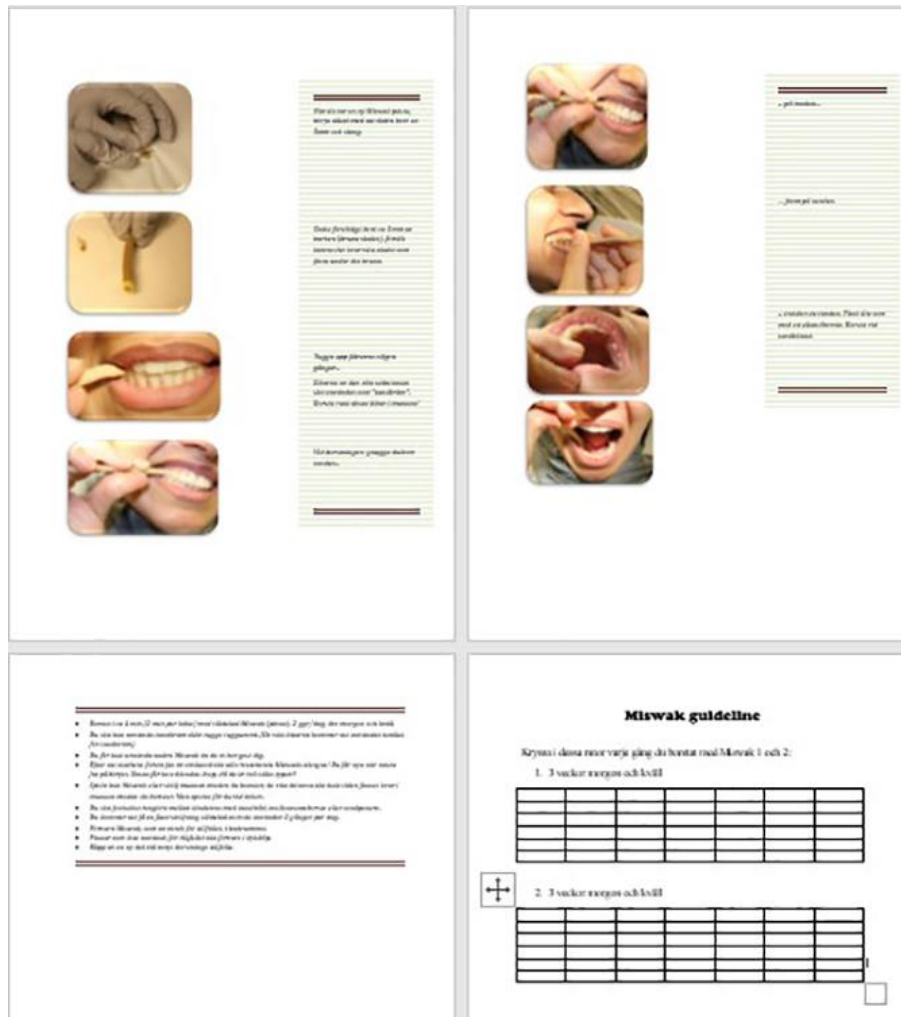


Fig. 3. Written instructions were given to the patients on how to use the Miswak with photos and a brushing schedule.

2.2.3 Clinical examination

Before the study began the clinicians, and dental hygienists were calibrated

The patients came from a government dental clinic in Stockholm, Sweden and the Dental Faculty at KI, Stockholm, Sweden.

Four clinical parameters were registered: a) Plaque index (PI), according to Silness & Loe 1964(63), b) Gingival index (GI) according to Silness & Loe 1963 and Loe, 1967)(64), C) Visual plaque index (VPI), d) Bleeding on probing scores (BoP, yes/no) at each of 4 sites per tooth.

PI and VPI were measured to assess both extension and amount of dental plaque..

Subgingival microflora Sterile paper-points were used to collect subgingival plaque and special type of paper strips used to collect GCF samples from four inflamed sites in the mouth, one in each quadrant (Figure 4). These samples were gathered in Eppendorf tubes containing 0.15 ml Tris-EDTA buffer (10 mmol/l Tris-HCL, 1.0 mmol/l EDTA + 0.10 ml NaOH, pH 7.6) and stored at -18°C to -80°C until further analysis. Samples were sent to the

microbiology laboratory at Forsythe Institute, Harvard, Boston, for analysis by checkerboard DNA-DNA Hybridization techniques with 41 predetermined bacterial species that were considered relevant to this research (65, 66).

Statistical analyses

The sample size calculation was based on a previous cross over study comparing Miswak and toothbrush in 15 participants, in which a significant difference in PI and GI between Miswak and toothbrush was found (Al-Otaibi et al., 2003)(49). Based on this study, the power calculation revealed a sample size of 25 in each group would have 80% power to detect a difference between groups in plaque index means of 0.19. The non-parametric Wilcoxon matched pairs test was applied to evaluate statistical differences between baseline and follow-up and between active and inactive Miswak. Statistical analysis was performed using the SPSS-Statistics version 20.0 (Stockholm, Sweden).

2.3 PAPER II

2.3.1 Collection and maintenance of plant material

Roots of *S. persica*, were purchased directly from a farmer in Jizan, Saudi Arabia and shipped to Karolinska Institutet, Stockholm, Sweden where they were manually cleaned, sorted, placed in air-tight polythene bags, and stored at -80 °C till used for essential oil extraction or using as chewing stick in different studies (Fig. 4).



Fig. 4. *Salvadora persica* L. roots “Miswak” harvested from Arak trees.

2.3.2 Extraction of essential oil

To extract the essential oil from the *S. persica* L. roots, 1.4 kg fresh twigs were cut into 20-40 mm long pieces and mixed with 700 mL double-distilled water. The resulting mixture was subjected to hydrodistillation for 5-6 h in a glass distillation apparatus (Fig. 5). The collected distillate (oil/water mixture) was extracted three times with HPLC grade hexane (VWR International, Sweden) with the help of a separating funnel. Anhydrous MgSO_4 (Alfa Aesar, UK) was added to the hexane extract to remove any traces of water. After filtration, hexane was evaporated using a rotary evaporator (Buchi Rotavapor- R210, Switzerland) at 20 °C under reduced pressure. The essential oil obtained was then weighed and the yield calculated as the percentage (w/w) of the total plant material. The EO was stored at -20 °C in a freezer till further experiments and analyses with GC-MS (Fig. 6).



Fig.5. Steam distillation set-up with Miswak roots at the Chemical Ecology Laboratory, KTH Royal Institutet of Technology in Stockholm, Sweden.



Fig. 6. Gas chromatography-mass spectrometry was used to separate and identify different compounds in the *Salvadora persica* essential oil

2.3.3 Collection of used Miswak and saliva samples

Twelve adults, 5 men and 7 women, with good oral health were chosen to participate in this study. The purpose of the study was fully explained, and informed consent was obtained from all participants before the study began commenced. The study was approved by the regional Research Ethics Committee in Stockholm (# 2012/609-31/1).

Each of the 12 participants was given a fresh chewing twig of the *S.persica* root (Miswak) that was 150 mm long with a diameter of about 10 mm. This twig was used throughout the entire study period. To expose fresh twig, each participant cut off a 2-mm long piece from one side of the twig. From the remaining twig, a circa 7-mm long piece of previously unexposed, fresh Miswak was cut. This piece was collected in a labelled Eppendorf tube so that the quantity of compounds originally present in each twig could be determined. After removal of the “baseline” piece, a 7-10-mm long piece of the twig was chewed, and the resulting fibrous brush was used to clean the teeth. During the cleaning process saliva from the participants was collected in a 15 mL Falcon tube (Fig. 7).

The used brush was then cut and placed in a labelled Eppendorf tube. The saliva and used twigs were collected after the first, second, and fourth use of the twig (Fig. 8). All the

Eppendorf and Falcon tubes were collected from the participants and stored in a freezer at - 80° C pending extraction and analysis of volatile compounds using (GC-MS).

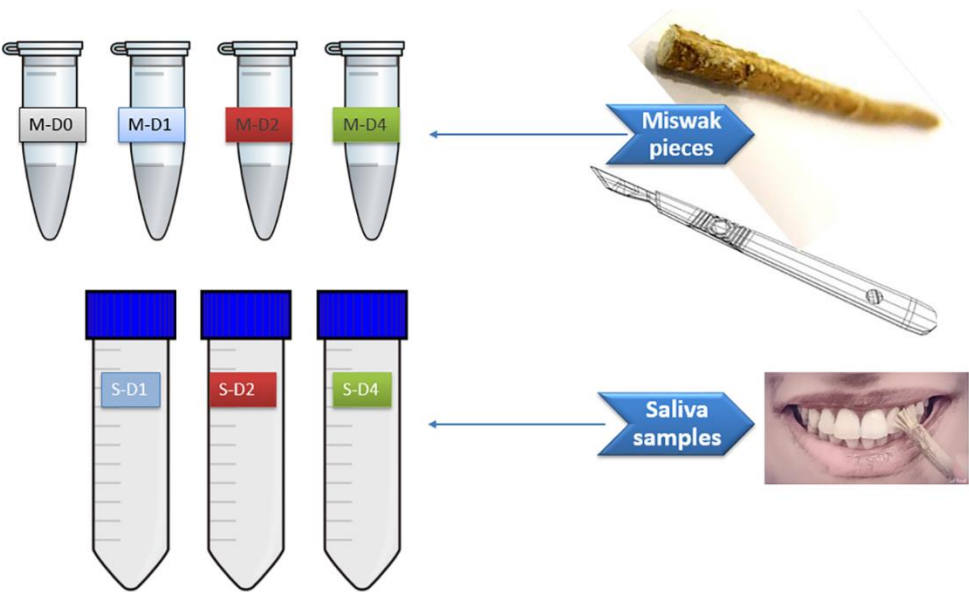


Figure 7. Sample collection protocol.

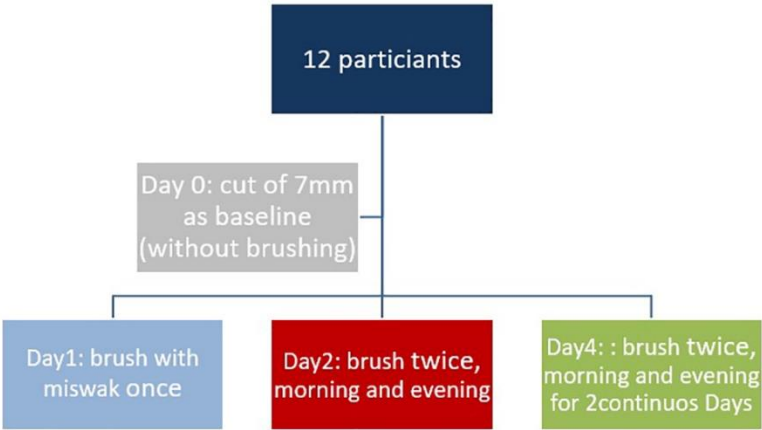


Figure 8. Chart illustrating the frequency of brushing with Miswak and collecting the saliva and the used brush tip afterwards.

2.3.4 Collection of volatiles from saliva

The frozen saliva samples were thawed by being kept at room temperature for 1.5 h and then for another 30 min so that the headspace (HS) air in the tubes could be equilibrated with volatile compounds in saliva (Fig. 9). After equilibration, the solid-phase microextraction (SPME) syringe was immersed in the sampling tube through a pinhole in the aluminum foil covering the mouth of the tube and the SPME fiber was exposed to the HS of the saliva for 2 h for the collection of volatiles. The SPME fiber was retracted to the syringe and immediately injected into the injector of the gas chromatograph. The SPME consisted of 65- μ m fibre with polydimethylsiloxane/divinylbenzene (PDMS/DVB) coating on a stable flex fibre (Supelco, USA). Prior to its first use the SPME was conditioned at 250 °C for 30 min.

2.3.5 Extraction of chemicals from fresh and used pieces of Miswak

Pieces of Miswak twigs, unused and used, were thawed for 1h at room temperature, cut into small pieces and placed in pre-weighed and labeled glass vials. For chemical extraction, the Miswak pieces were dipped in 1 ml hexane for 24 h at room temperature. The extracts were collected in fresh glass vials using a glass Pasteur pipette. The residual Miswak pieces were rinsed with another 500 μ l pure hexane and the extracts were pooled in the collection vials. Following chemical extraction, the Miswak pieces were dried in an oven at 80°C for 15 h. After 15 h, their dry mass was measured after keeping the pieces in desiccator to obtain constant mass. Hexane extracts of Miswak pieces were injected (1 μ l) into the GC-MS via liquid injection using a syringe.

2.3.6 Chemical analyses of saliva and pieces of Miswak

Volatile compounds collected on SPME fibre and extracted in hexane were separated and identified in the GC-MS (Fig.6) using a Varian 3400 GC (Varian, Palo Alto, CA, USA) connected to a Finnigan SSQ 7000 (Waltham, MA, USA) quadruple mass spectrometer with electron ionisation. The GC was equipped with a split/split less injector (split less mode, 30 sec); the carrier gas was helium (99.99%, Strandmöllen AB, Ljungby, Sweden), at a constant pressure of 10 psi. The GC was fitted with a DB-Wax capillary column (30 m, 0.25 mm ID, and 25 μ m film thickness, J & W Agilent, Santa Clara, CA, USA). The temperature program of the GC oven was 45 °C for 30 sec. increasing in temperature at a rate of 8 °C min⁻¹ till 235 °C and maintained at 235 °C for 5.75 min. The injector temperature was isothermally set at 230 °C and the transfer line connecting the GC to the MS was isothermally set at 235 °C. The MS ion source temperature was 150 °C; mass spectra were obtained at 70 eV with a mass range of 30 m/z to 400 m/z in positive centroid mode. Separated compounds were initially identified by comparing their mass spectra to the NIST-08 (National Institute of Standard and Technology, USA) MS library. The final authentication was made by analysing pure standard compounds on GC-MS using the same parameters as used for the Miswak and saliva samples. Quantification of identified compounds was made by running different concentrations of compounds on GC-MS followed by producing standard curve.



Fig. 9.Collection of volatiles from Saliva using Solid phase microextraction device (SPME).

2.3.7 Salivary retention after brushing with *S. persica* sticks

Three volunteers were assigned a fresh Miswak stick and asked to collect saliva before brushing, immediately after brushing and 5, 10, and 30 min after brushing. The saliva samples were treated as previously described until analyzed for the quantification of BITC using SPME GC-MS.

2.3.8 Cytotoxicity of Miswak essential oil and the major components

S. persica EO or pure components BITC, BA, and BC stock solutions (1mg/ml) were prepared by dissolving them in absolute ethanol. The solutions were sterilized by filtering them through a 0.22 μ m filter and storing them at -20° C until use for cytotoxicity bioassay. Working concentrations of essential oil or pure compounds were made in Dulbecco's modified Eagle's medium (DMEM) by diluting the respective stock solutions mentioned above.

The cytotoxic effect of Miswak oil and other chemical compounds on cell viability was evaluated by determining metabolically active fibroblasts using a colorimetric procedure described by Mosmann(67), and that applied by methyl tetrazolium (MTT) cell viability assay kit according to the manufacturer's instruction (Abnova, Taipei, Taiwan).

2.3.9 Cell cultures

Primary hGFs were established from gingival biopsies obtained from healthy patients with no clinical signs of periodontal disease. The Ethics Committee at Huddinge University

Hospital approved the protocol, including the collection of gingival biopsies, (#377/98). The hGFs were established and cultured in the manner previously described (68). The hGFs used for the experiments were cultured in 175 cm² tissue culture flasks until reaching 70-80% confluence and the experiments were performed between the 7th and 14th passages.

Immortalized normal human Oral Keratinocyte (OKF6/TERT-2) cells (69) were cultured in EpiLife[®] cell culture medium (Invitrogen, Carlsbad CA, USA) supplemented with 0.06 mM calcium, 10 µg/ml gentamicin, 0.25 µg/ml amphotericin B and defined growth serum (EDGS) (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

2.3.10 Cell viability bioassay

The hGFs (1×10^4) were seeded in 96-well plates in tetraplicate manner in DMEM supplemented with penicillin (50 U/ml), streptomycin (50 µg/ml) and fetal calf serum (FCS) (5%) and cultured at 37 °C for 24 h. The cell layers were rinsed twice with serum-free DMEM followed by the addition of 0.1 ml serum-free medium containing different concentrations (0.4µg/ml to 3µg/ml) of Miswak essential oil, BITC, BC, and BA. Control cells were treated with corresponding amounts of ethanol added in samples or serum-free DMEM only. After an incubation period of 24 h, MTT cell viability assay kit (Abnova, Taipei, Taiwan) was used to assess the viability of gingival fibroblast according to the manufacturer instructions. The hGFs were incubated with MTT reagent for 4 h at 37° C, after which solubilizing solution was added. The resulting mixture was shaken for 1 h at room temperature and read at λ_{\max} 507nm on an ELISA reader.

2.3.11 Effect of compound exposure time on cell viability

hGFs were seeded in a 96-well microtiter plate as previously mentioned, incubated for 24 h, washed with a serum-free medium, and then treated with Miswak EO, BITC, BC, or BA. The concentration of Miswak EO and BITC ranged from 1 µg/ml to 100µg/ml, whereas concentrations for BC and BA ranged from 0.5 µg/ml to 20 µg/ml. After an exposure time of either 10 or 30 min, the cells were washed twice with a serum-free medium and 0.1 ml of complete medium was added to the cells. They were then incubated for another 24 h. Cell-recovery assessment was carried out using the MTT kit described above.

The immortalized normal human Oral keratinocytes were seeded in 96-well plates until reaching 80% confluence, after which the medium was then removed and replaced with 100µl of medium with increasing concentrations of either Miswak EO or BITC (5-100 µg/ml) and incubated for 24 h. Cell viability was detected by adding MTT to the medium, incubating it for 4 h, and then adding dimethyl sulfoxide (DMSO) to dissolve the formazan product.

After the plate was shaken at low speed for 10 minutes optical density was measured at 540 nm using an ELISA reader.

Results are reported as mean of three experiments with four replicates in each experiment for each concentration.

2.3.12 Antimicrobial effect of Miswak essential oil and pure compounds.

2.3.13 Bacterial isolates and growth conditions.

The antibacterial effect of Miswak essential oil and major components was tested on the following bacteria: *Aggregatibacter actinomycetemcomitans* KH 1519, *Haemophilus influenzae*, ATCC 49247 and *Porphyromonas gingivalis*, ATCC 33277.

H. influenzae was propagated overnight in chocolate agar plates in a 5% CO₂ incubator. *A. actinomycetemcomitans* was propagated on blood agar plates for two days in 5% CO₂ atmosphere. *P. gingivalis* was propagated for 6 days on Brucella agar plates supplemented with Hemin (0.05 mg/ml), vitamin K (0.01 mg/ml) and citrated horse blood (5%) in an anaerobic atmosphere created with gas-packs (GasPak™, Becton Dickinson, Franklin Lake, NJ, USA). All agar plates were prepared and retrieved from the substrate unit, at the clinical microbiology laboratory, Karolinska Hospital, Huddinge, Sweden.

2.3.14 Antibacterial dose-response assay

Bacterial colonies of *H. influenzae*, *A. actinomycetemcomitans*, and *P. gingivalis* were suspended in 2 mL of 0.9 % NaCl solution and optical density was set to 0.5 at a wavelength of 590 nm. Prior to the test, all bacterial suspensions were further diluted 10⁴-fold in fresh saline medium.

The Miswak EO, BITC, BA, and BC dilutions were prepared by dissolving in DMSO. 5 µl of EO, pure compounds, or their diluted solution in DMSO were added to 495 µl of 10⁴ fold diluted bacterial suspension to produce the following dose concentrations: 1%, 0.1%, 0.05%, 0.02%, 0.01%, 0.002%, and 0.001%. In all experiments a negative control of 5 µl DMSO, and a positive control of 5 µl (0.05 %) of CHX was used. The suspension mixture was spread on agar plates with a respective growth medium after 10 min of incubation at room temperature. The plates of *H. influenzae* were incubated for 24 h, *A. actinomycetemcomitans* for 2 days, and *P. gingivalis* for 5-6 days. After incubation the bacterial colonies on each plate were counted manually. All tests were performed at least 3 times.

2.3.15 Statistical Analysis:

Data of this study are presented as means and standard deviations of at least three separate experiments with two to three replicates. The two-tailed paired *t*-tests were used to examine the significance of differences between control and test samples. The one-way ANOVA with Bonferroni post hoc test was used to find difference between bacterial growth when they were treated with same concentration of Miswak EO or pure compounds. The statistical analyses were carried out using SPSS 20.0 computer software (IBM, USA).

2.4 PAPER III

2.4.1 *Salvadora persica* oil and BITC

S. persica L. twigs were cut into 20-30-mm long pieces and mixed with 700 ml double-distilled water. The resulting mixture was subjected to hydrodistillation for 5-6 h in a glass distillation apparatus. The collected distillate (oil/water mixture) was extracted three times with HPLC grade hexane (VWR International, Sweden) and a separating funnel. Anhydrous MgSO_4 (Alfa Aesar, UK) was added to the hexane extract to remove any traces of water. After filtration, hexane was evaporated using a rotary evaporator (Buchi Rotavapor- R210, Switzerland) at 20 °C under reduced pressure. The essential oil obtained was then weighed and the yield calculated as percentage (w/w) of the total plant material. The essential oil was stored till used at -20°C in a freezer (Fig. 5).

2.4.2 Cell culture

hGFs cultures were established from gingival biopsies obtained from two systemically and periodontally healthy subjects (aged 7–12y) as previously described (68). The Swedish Central Ethical Review Board, Stockholm approved the collection of biopsies and the establishment of the fibroblast cells (Dnr. 377/98).

Cells were grown in DMEM supplemented with 5% fetal calf serum (FCS) (Invitrogen Life Technologies, Scotland, UK), 1% penicillin (50 units/ml), streptomycin (50 µg/ml) at 37°C with 5% CO_2 . hGFs at passages 7-14 were used in all experiments to insure stability.

Immortalized normal human oral keratinocytes (OKF6/TERT-2) cells (69) were cultured in EpiLife[®] cell culture medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0.06 mM calcium, 10 µg/ml gentamicin, 0.25 µg/ml amphotericin B and defined growth serum (EDGS) (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed after 1 day and subsequently every 2–3 days until the cells reached 70-80% confluence.

Based on our earlier tests of the cytotoxic effect on Gingival fibroblasts and oral keratinocytes (70) we selected subcytotoxic concentrations for testing the anti-inflammatory effect in this study.

For experiments, hGFs and OK were seeded in 24-well plates and treated when reaching 80% confluence with subtoxic concentrations of Miswak oil (0,25-1,0 µg/ml), BITC (0,25-1,0 µg/ml), or IL-1 β (300 pg/ml, BioLegend, San Diego, CA, USA) alone or in combination with Miswak EO and IL-1 β or BITC and IL-1 β for 24 h. Supernatants were collected at 24 h and stored at -80 °C pending analysis. All experiments were performed in triplicate and repeated at least two times.

2.4.3 Enzyme Linked Immunosorbent Assay (ELISA)

The levels of IL-6, IL-8, and MMP-1 were determined in cell supernatants by ELISA according to the instructions provided by the manufacturer (R&D Systems Inc; Minneapolis, MN, USA). In hGFs stimulated with IL-1 β alone or in combination with Miswak EO or BITC undiluted samples were above the standard point with the highest concentration of IL-6, IL-8, and MMP-1, so those samples were re-run in dilutions 1:40, 1:10, and 1:10 respectively. The ranges of the kits were 9.38-600 pg/ml, 31.2-2000 pg/ml, and 156.0-10000 pg/ml for IL-6, IL-8, and MMP-1 respectively.

2.4.4 Statistical Analysis

Comparisons between groups were assessed by one-way ANOVA combined with Tukey's post hoc test. The data are presented as the mean \pm standard error of the mean (SEM) and *P*-values of <0.05 were considered significant.

3 RESULTS

3.1 PAPER I

Twenty-four out of 28 participants completed the study between Jan/2013-June/2013. Four dropped out, one because of family matters, other was due to antibiotic consumption and two dropped out after the first visit with the cleaning without giving an explanation.

The clinical examination showed that the Miswak and the toothbrush did not differ in efficacy. There was no difference in dental plaque between baseline and the three weeks follow-up, neither in the extension, (VPI) nor in the amount of plaque (PI). There were also no differences between the active and inactive Miswak at follow-up. The extent (BoP) and severity (GI) of gingival inflammation did not differ between baselines and follow-ups, or between active and inactive Miswak (Table 1).

The microbial analysis showed no significant differences after use Active or Inactive Miswak. However, comparing baseline with follow-up, 16 species of bacteria showed increase ($p < 0.05$) after the use of inactive Miswak while no species showed a similar change after the use of active Miswak (Table 2). The following bacteria: *Actinomyces naeslundii*, *Eubacterium saburreum*, *Actinomyces oris*, *Actinomyces gerencseriae*, *Streptococcus oralis*, *Actinomyces israelii*, *Actinomyces odontolyticus*, *Streptococcus gordonii*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Capnocytophaga ochracea*, *Neisseria mucosa*, *S.gingivalis*, *Selenomonas noxia*, *Eikenella corrodens* and *Capnocytophaga sputigena* showed increased levels ($p < 0.05$) after the use of inactive Miswak. Eight of these species were Gram-negative bacteria (*A.a*, *F.nucleatum*, *C.ochracea*, *N.mucosa*, *S.gingivalis*, *S.noxia*, *E.corrodens* and *C.sputigena*). The remaining 8 bacterial species (*A.naeslundii*, *E.saburreum*, *A.oris*, *A.gerencseriae*, *S.oralis*, *A.israelii*, *A.odontolyticus*, *S.gordonii*) were Gram-positive. At follow-up, 19 species had lower values after use of active Miswak and 3 had higher compared to inactive. None of these differences had a p -value < 0.05 . Moreover, none of the participants reported any harmful side effects during the trial.

Table 1. Dental plaque, registered as amount of (PI), extent of (VPI), gingival inflammation registered as severity (GI), and extent of (BoP) for 24 participants before and after use of inactive and active Miswak . The findings are expressed as medians and interquartile ranges (IR)

All sites	Inactive			Active		
	Median (IR)			Median (IR)		
	Baseline	Follow up	Change	Baseline	Follow up	Change
PI	1.3 (0.7)	1.4 (0.8)	0.0 (0.3)	1.6 (0.7)	1.5 (1.0)	0.0 (0.4)
VPI (%)	20.2 (25.0)	29.7 (28.5)	4.4 (13.5)	25.4 (34.5)	21.1 (28.0)	-2.5 (15.6)
GI	2.1 (0.5)	2.0 (0.2)	0.1 (0.4)	2.0 (0.5)	2.0 (0.4)	0.0 (0.5)
BoP (%)	17.4 (13.2)	22.0 (12.7)	1.3 (10.7)	17.5 (11.8)	18.4 (18.2)	0.0 (16.8)

Table 2 Median value and interquartile range for the 40 tested bacteria expressed as 105 bacteria. Significance of differences between active and inactive Miswak sticks were calculated with Wilcoxon signed rank test. N=24

Bacteria	Gram staining	Baseline Inactive	Follow-up Inactive	Change Inactive	p-value 1 Inactive	Baseline Active	Follow-up Active	Change Active	p-value 2 Active	p-value 3 Difference
<i>Actinomyces naeslundii</i>	+	0.10 (0.20)	0.20 (0.03)	0.10 (0.20)	0.022	0.10 (0.17)	0.10 (0.18)	0.00 (0.10)	-	-
<i>Streptococcus constellatus</i>	+	0.10 (0.10)	0.10(0.10)	0.00 (0.10)	-	0.10 (0.10)	0.10 (0.10)	0.00 (0.08)	-	-
<i>Eubacterium nodatum</i>	+	0.10 (0.43)	0.10 (0.25)	0.10 (0.40)	-	0.10 (0.55)	0.10 (0.65)	0.00 (0.20)	-	-
<i>Porphyromonas gingivalis</i>	-	0.20 (1.38)	0.30 (1.05)	0.10 (0.10)	-	0.10 (3.30)	0.20 (3.00)	0.05 (0.55)	-	-
<i>Aggregatibacter actinomycetemcomitans</i>	-	0.05 (0.10)	0.10 (0.15)	0.10 (0.10)	0.028	0.10 (0.18)	0.10 (0.10)	0.00 (0.18)	-	-
<i>Fusobacterium nucleatum sub spp. vincentii</i>	-	0.70 (1.55)	1.50 (1.70)	0.30 (1.15)	0.028	0.90 (1.6)	0.70 (1.85)	0.00 (0.08)	-	-
<i>Campylobacter rectus</i>	-	0.05 (0.10)	0.10 (0.20)	0.00 (0.20)	-	0.05 (0.20)	0.05 (0.10)	0.00 (0.08)	-	-
<i>Treponema socranskii</i>	-	0.10 (0.20)	0.10 (0.20)	0.10 (0.20)	-	0.10 (0.20)	0.10 (0.43)	0.00 (0.18)	-	-
<i>Eubacterium saburreum</i>	+	0.20 (0.10)	0.30 (0.55)	0.10 (0.50)	0.035	0.20 (0.20)	0.20 (0.38)	0.00 (0.30)	-	-
<i>Parvimonas micra</i>	+	0.20 (0.20)	0.20 (0.30)	0.00 (0.20)	-	0.10 (0.57)	0.15 (0.35)	0.00 (0.28)	-	-
<i>Veillonella parvula</i>	-	0.15 (0.43)	0.50 (0.90)	0.10 (0.50)	-	0.20 (0.28)	0.40 (0.72)	0.10 (0.48)	-	-

<i>Actinomyces oris</i>	+	0.10 (0.12)	0.20 (0.30)	0.10 (0.25)	0.012	0.10 (0.10)	0.20 (0.20)	0.05 (0.20)	-	0.029
<i>Streptococcus anginosus</i>	+	0.00 (0.10)	0.10 (0.10)	0.00 (0.10)	-	0.05 (0.10)	0.00 (0.10)	0.00 (0.00)	-	0.012
<i>Streptococcus sanguinis</i>	+	0.05 (0.10)	0.10 (0.20)	0.00 (0.10)	-	0.10 (0.10)	0.10 (0.10)	0.00 (0.15)	-	0.012
<i>Actinomyces gerencseriae</i>	+	0.10 (0.10)	0.20 (0.02)	0.10 (0.15)	0.034	0.10 (0.18)	0.10 (0.25)	0.00 (0.10)	-	-
<i>Streptococcus oralis</i>	+	0.00 (0.10)	0.10 (0.15)	0.00 (0.10)	0.013	0.05 (0.10)	0.10 (0.10)	0.00 (0.10)	-	-
<i>Capnocytophaga ochracea</i>	-	0.25 (0.25)	0.50 (0.70)	0.10 (0.55)	0.033	0.35 (0.47)	0.35 (0.60)	0.00 (0.38)	-	0.029
<i>Actinomyces israelii</i>	+	0.10 (0.03)	0.20 (0.10)	0.10 (0.10)	0.047	0.10 (0.10)	0.10 (0.10)	0.00 (0.18)	-	0.040
<i>Sorbus intermedia</i>	+	0.00 (0.10)	0.10 (0.10)	0.10 (0.10)	-	0.10 (0.00)	0.10 (0.10)	0.00 (0.10)	-	0.014
<i>Treponema denticola</i>	-	0.25 (0.95)	0.30 (0.75)	0.00 (0.90)	-	0.30 (1.48)	0.40 (1.30)	0.00 (0.53)	-	-
<i>Prevotella nigrescens</i>	-	0.65 (0.90)	1.00 (1.95)	0.10 (1.45)	-	0.55 (0.1.58)	0.60 (1.65)	0.00 (2.00)	-	-
<i>Actinomyces odontolyticus</i>	+	0.10 (0.13)	0.20 (0.20)	0.10 (0.20)	0.016	0.15 (0.10)	0.10 (0.17)	0.00 (0.20)	-	0.040
<i>Fusobacterium nucleatum</i>	-	0.75 (1.00)	1.20 (1.30)	0.10 (0.95)	-	0.90 (1.37)	0.95 (1.52)	0.00 (1.43)	-	-
Subspecies Polymorphum										
<i>Campylobacter showae</i>	-	0.10 (0.10)	0.10 (0.30)	0.00 (0.30)	-	0.15 (0.15)	0.10 (0.38)	0.00 (0.20)	-	-
<i>Fusobacterium periodonticum</i>	-	0.40 (0.65)	0.70 (0.70)	0.10 (0.55)	-	0.60 (0.85)	0.50 (0.80)	0.05 (0.72)	-	-
<i>Neisseria mucosa</i>	-	0.35 (0.60)	0.80 (1.05)	0.30 (0.80)	0.018	0.45 (0.83)	0.95 (1.38)	0.10 (1.30)	-	-
<i>Fusobacterium nucleatum</i>	-	0.85 (1.60)	1.00 (1.80)	0.20 (0.85)	-	1.00 (2.02)	0.80 1.85)	0.05 (1.13)	-	-
Subspecies nucleatum										

<i>Capnocytophaga gingivalis</i>	-	0.10 (0.13)	0.40 (0.40)	0.00 (0.35)	0.043	0.20 (0.28)	0.20 (0.38)	0.00 (0.30)	-	-
<i>Streptococcus gordonii</i>	+	0.00 (0.10)	0.10 (0.10)	0.00 (0.10)	0.049	0.10 (0.10)	0.05 (0.10)	0.00 (0.10)	-	0.026
<i>Tannerella forsythia</i>	-	0.60 (1.20)	0.30 (1.05)	0.10 (1.05)	-	0.45 (1.35)	0.60 (2.27)	0.00 (1.38)	-	-
<i>Selenomonas Noxia</i>	+	0.15 (0.15)	0.30 (0.60)	0.10 (0.40)	0.022	0.20 (0.40)	0.30 (0.38)	0.00 (0.38)	-	0.045
<i>Propionibacterium acnes</i>	+	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	-	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	-	-
<i>Prevotella Melaninogenica</i>	-	0.10 (0.13)	0.10 (0.15)	0.10 (0.10)	-	0.10 (0.20)	0.10 (0.35)	0.00 (0.20)	-	-
<i>Streptococcus mitis</i>	+	0.10 (0.13)	0.10 (0.15)	0.00 (0.20)	-	0.10 (0.10)	0.10 (0.10)	0.00 (0.10)	-	-
<i>Eikenella corrodens</i>	-	0.30 (0.43)	0.60 (0.65)	0.20 (0.80)	0.026	0.50 (0.55)	0.50 (0.90)	0.00 (0.78)	-	-
<i>Gemella morbillorum</i>	+	0.10 (0.10)	0.10 (0.10)	0.00 (0.10)	-	0.10 (0.15)	0.10 (0.20)	0.00 (0.20)	-	-
<i>Capnocytophaga sputigena</i>	-	0.20 (0.20)	0.30 (0.35)	0.10 (0.35)	0.005	0.20 (0.30)	0.25 (0.40)	0.05 (0.30)	-	0.045
<i>Leptotrichia buccalis</i>	±	0.20 (0.02)	0.30 (0.25)	0.00 (0.25)	-	0.20 (0.28)	0.20 (0.28)	0.00 (0.20)	-	-
<i>Campylobacter gracilis</i>	+	0.10 (0.10)	0.10 (0.20)	0.00 (0.15)	-	0.10 (0.18)	0.10 (0.10)	0.00 (0.20)	-	-
<i>Prevotella intermedia</i>	-	0.20 (0.55)	0.30 (0.70)	0.1 (0.04)	-	0.20 (0.85)	0.30 (0.68)	0.00 (0.68)	-	-

p-value 1 indicate significance of the difference between baseline and follow-up after use of the inactive Miswak

p-value 2 indicate significance of the difference between baseline and follow-up after use of the active Miswak

p-value 3 indicate significance of the difference between baseline and follow-up after use of the inactive Miswak

3.2 PAPER II

3.2.1 Chemical analysis of *S. persica* essential oil

The hydro-distillation of *S. persica* roots twigs produced 1.4 g of essential oil giving rise 0.001% yield. GC-MS analysis of the oil revealed the presence of three major compounds constituting 92% of the oil (Table 3). The main compounds in *S. persica* EO were BITC, (CID: 2346), 74.4 % followed by BC (CID: 8794), 16.3 %, and BA (CID: 240), 0.7 % (Table 3).

Table 3. Chemical composition of *S. persica* roots essential oil and the amount of benzyl isothiocyanate, benzyl cyanide and benzaldehyde in Miswak brushes.

Sample	Benzyl isothiocyanate	Benzyl cyanide	Benzaldehyde
Essential oil	74.42%	16.26%	0.67%
Amount of compounds in Miswak brushes (mg/g dry wt)			
Miswak-0	6.49 ± 1.14 ^a	0.190 ± 0.036 ^a	0.005 ± 0.001 ^a
Miswak-1	3.52 ± 0.66 ^b	0.129 ± 0.034 ^b	0.006 ± 0.002 ^a
Miswak-2	2.01 ± 0.42 ^c	0.072 ± 0.016 ^c	0.007 ± 0.002 ^a
Miswak-4	1.49 ± 0.51 ^c	0.037 ± 0.012 ^d	0.005 ± 0.001 ^a

Miswak-0 stands for baseline piece, that is, the amount of compounds present originally in the Miswak pieces. Miswak-1 stands for amount of compounds found in Miswak brushes after one-time use; similarly Miswak-2 and Miswak-4 stand for amount of compounds in Miswak brushes after two- and four-time use. Different letters on values are indicating statistical difference ($p < 0.05$) between values presented in the same column. Each value expressed in the table is a mean ± standard error ($N = 12$).

3.2.2 Quantitative analysis of Miswak pieces and saliva

The amount of BITC found in unused Miswak pieces (base line) was 648.8 µg /100mg of Miswak, this amount was statistically different ($p > 0.05$) from the amount of BITC found in Miswak brushes after one, two and four times use (Table 3). Moreover, the BITC amount in Miswak-1 brush was significantly more ($p > 0.05$) than Miswak-2 and Miswak-4 brushes, however, Miswak-2 and Miswak-4 exhibited similar amount ($p > 0.05$). The amount of BC in all the four Miswak brushes was different ($p > 0.05$) whereas BA contents were statistically same (Table 3).

The mean amount of BITC content in saliva samples after one time use of the Miswak brush was 13.85 µg/ml whereas the amount decreased drastically to 1.65 µg/ml and 0.5 µg/ml after the same Miswak brush was used two times and four times respectively (Fig. 10a). BITC and

BC contents were only statically different ($p < 0.05$) when fresh Miswak was used to clean the teeth (Fig. 10a). When the previously used Miswak was used the amount of BITC and BC was very low compared with saliva-1.

3.2.3 Salivary retention after brushing with *S. persica* Miswak

The concentration of BITC in saliva immediately after using fresh Miswak was $13.8\mu\text{g}$. However, there was a marked reduction over time in the amount of all compounds (BITC, BC, BA) found in the saliva, and it disappeared completely after 5 min (Fig. 10b).

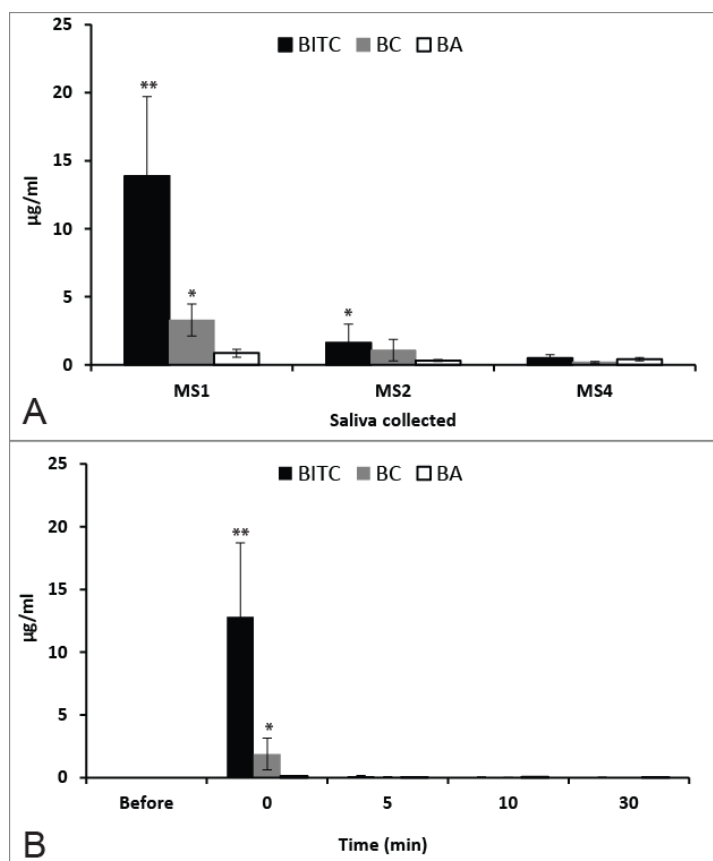


Fig. 10. Benzyl isothiocyanate (BITC), benzyl cyanide (BC) and benzaldehyde (BA) concentration in saliva. Fig. 9A. The concentration of compounds in saliva collected during fresh Miswak use (MS1), saliva collected when Miswak used second time (MS-2) and saliva collected while using Miswak fourth time (MS4). Fig. 9B. The concentration of compounds in saliva before brushing, immediately after brushing with fresh Miswak and after 5, 10 and 30 min. In the above figure the bars denoted with * and ** are significantly different when the comparison was made between the concentration of same compound detected in different samples.

3.2.4 Cytotoxicity

3.2.5 Human Gingival fibroblasts hGFs

A 10-min exposure to 1–5 $\mu\text{g/ml}$ of Miswak oil or BITC did not affect the cell viability of hGFs compared to the negative control. A significant decrease in cell viability was observed at concentration of 10 $\mu\text{g/ml}$ or higher ($p > 0.05$) (Fig. 11). The cells exposed to 2.5 $\mu\text{g/ml}$ CHX showed similar viability as control cells ($p > 0.05$). The most cytotoxic compound was BA that decreased the cell viability even at lower concentration 0.5 $\mu\text{g/ml}$ (Fig. 9). However, there was no significant effect of a 10-min exposure to 0.5–5 $\mu\text{g/ml}$ of BC except for 10–20 $\mu\text{g/ml}$ ($p > 0.05$) (Fig. 11).

A significant decrease of cell viability was noted after a 24h exposure to Miswak oil at concentration 1.4 $\mu\text{g/ml}$ or higher ($p > 0.05$), and to BITC at concentrations ≥ 1 $\mu\text{g/ml}$ ($p > 0.05$) (Fig. 12). Twenty-four hours exposure to BC (0.4–3 $\mu\text{g/ml}$) showed no significant effect on cell viability whereas BA showed significant cell toxicity at concentrations of 0.8 $\mu\text{g/ml}$ or higher (Fig.12).

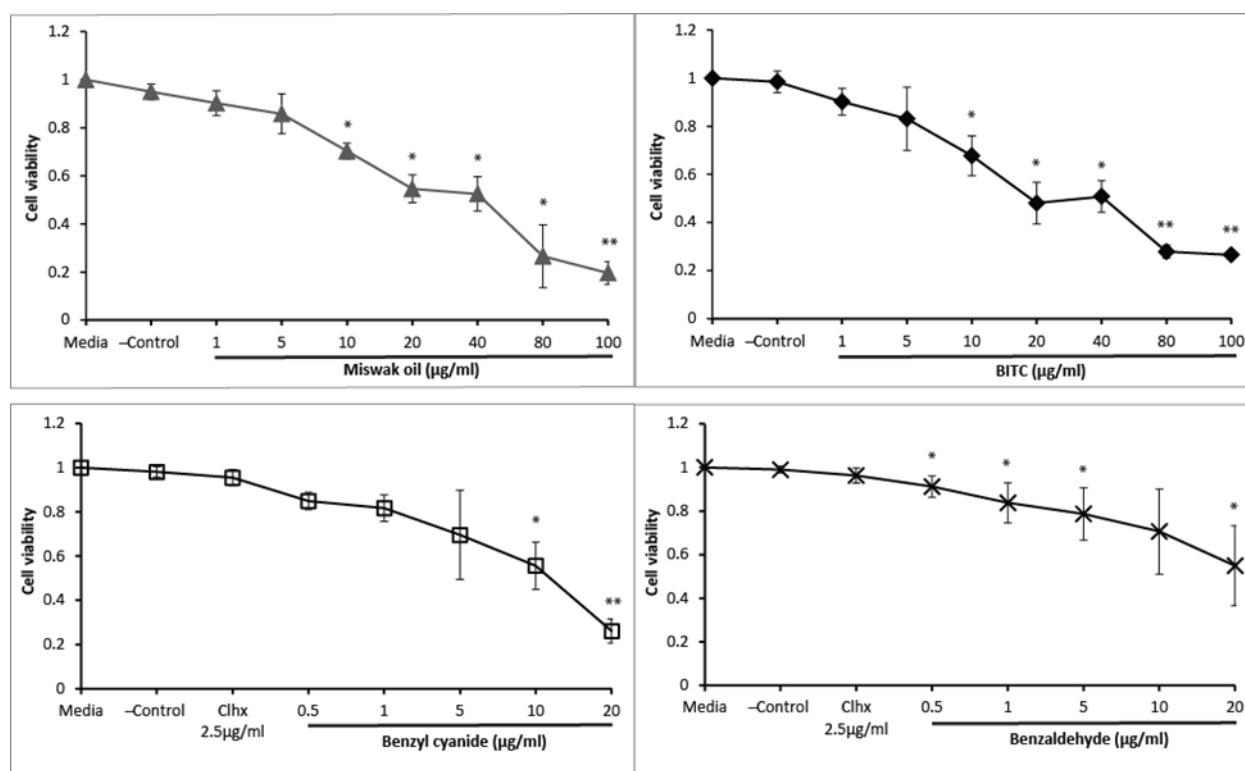


Fig. 11. Gingival fibroblasts cells exposed for 10 min to different concentrations of Miswak oil, benzyl isothiocyanate (BITC), benzyl cyanide, benzaldehyde and 2.5 $\mu\text{g/ml}$ of chlorhexidine. The bar denoted with * ($p < 0.05$), ** ($p < 0.01$) are significantly different from negative control (DMSO) and media control.

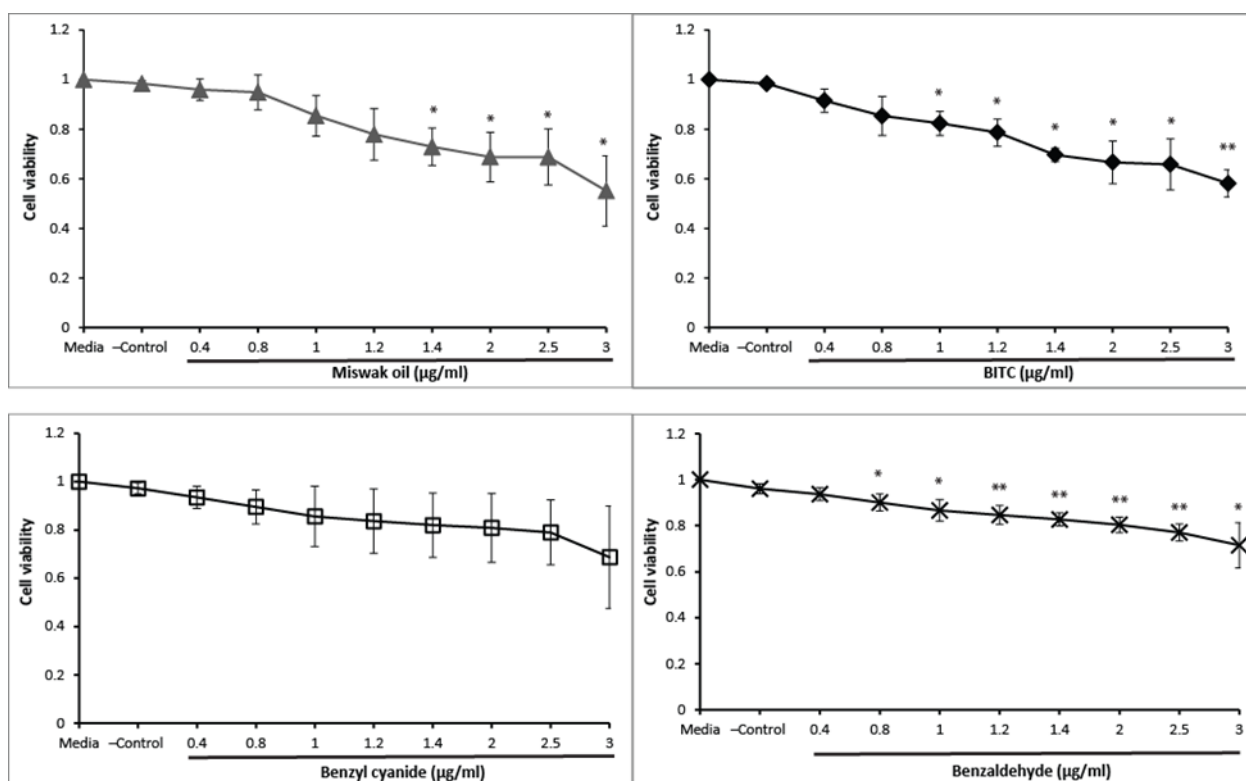


Fig.12. Gingival fibroblasts cell exposed for 24 h to different concentrations of Miswak essential oil, benzyl isothiocyanate (BITC), benzyl cyanide and benzaldehyde. The bars denoted with * ($p < 0.05$), ** ($p < 0.01$) are significantly different from negative control (DMSO) and media control.

3.2.6 Oral Keratinocytes

Miswak oil and BITC did not show any bad effect on the viability of keratinocytes when they were stimulated with Miswak oil or BITC for 24 h at the concentrations (5–100 µg/ml), however, at higher concentration they show better cell viability (Fig. 13) than control ($p < 0.05$).

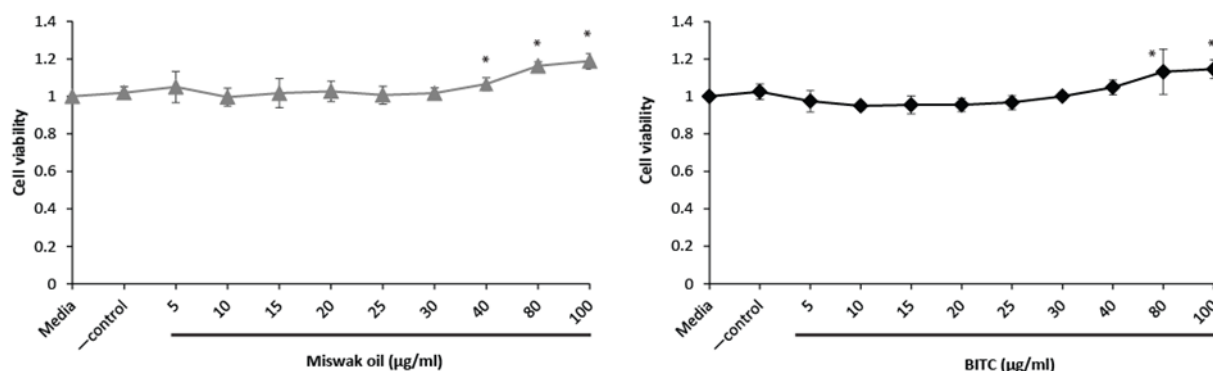


Fig. 13. Oral keratinocytes exposed for 24h to different concentrations of Miswak oil and benzyl isothiocyanate (BITC). The points indicate cell viability in relation to the viability using media alone. Points marked with * indicate a statistical difference from media control ($p < 0.05$).

3.2.7 Antimicrobial effect of Miswak essential oil and pure compounds

Miswak essential oil and BITC showed a dose-dependent antibacterial activity against the three bacteria tested (Fig 14, 15). *H. influenzae* and *P.gingivalis* were more susceptible towards Miswak essential oil thus their growth significantly inhibited ($p < 0.01$) at lowest concentration (11.7 $\mu\text{g/ml}$) tested in experiments (Fig. 14). In the presence of Miswak essential oil the growth of three tested bacteria was different ($p < 0.05$) from each other (Fig. 14). In contrast to Miswak oil the BITC only inhibited the growth of *P. gingivalis* at lowest concentration (Fig. 15). CHX completely inhibited the growth of all tested bacterial species at a concentration 500 $\mu\text{g/ml}$ (Fig 14, 15). BA showed better activity than BC against bacterial strains, however, both compounds were not able to inhibit the bacterial growth at concentration found in saliva or Miswak brushes (Fig. 16, 17).

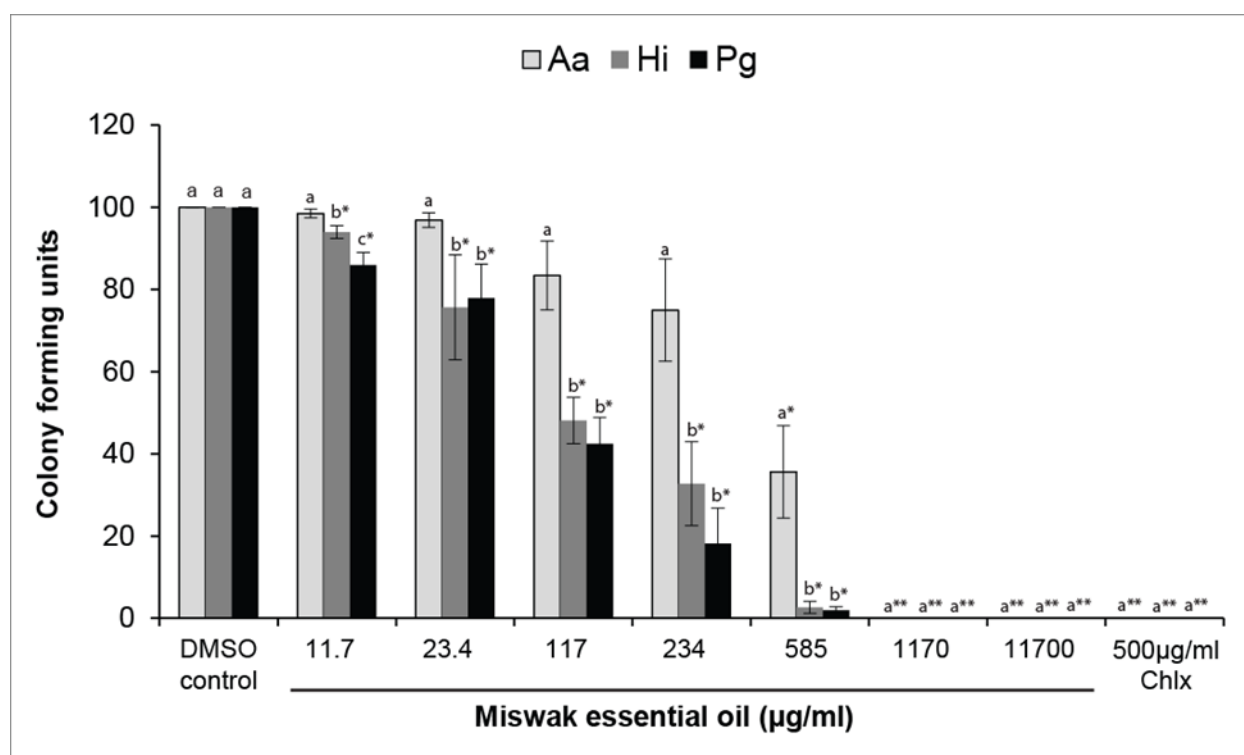


Fig. 14. Inhibition of growth of *A. actinomycetemcomitans* (Aa), *H. influenzae* (Hi) and *P. gingivalis* (Pg). The individual bars present the percentage of colony forming units (CFU) at various concentrations of Miswak oil or chlorhexidine (Chlx) compared to the negative control (DMSO), set to 100%. In the above figure * stands for $p < 0.01-0.001$ and ** stands for $p < 0.0001$ when number of CFUs were compared between negative control and different concentrations of Miswak essential oil or Chlx. Different letters on the bars presenting significant difference ($p < 0.05$) between the CFU of three bacteria tested at a given concentration of Miswak essential oil or Chlx independently, **b** indicate a significant difference compared to **a**, **c** indicate a difference compared to both **a** and **b**.

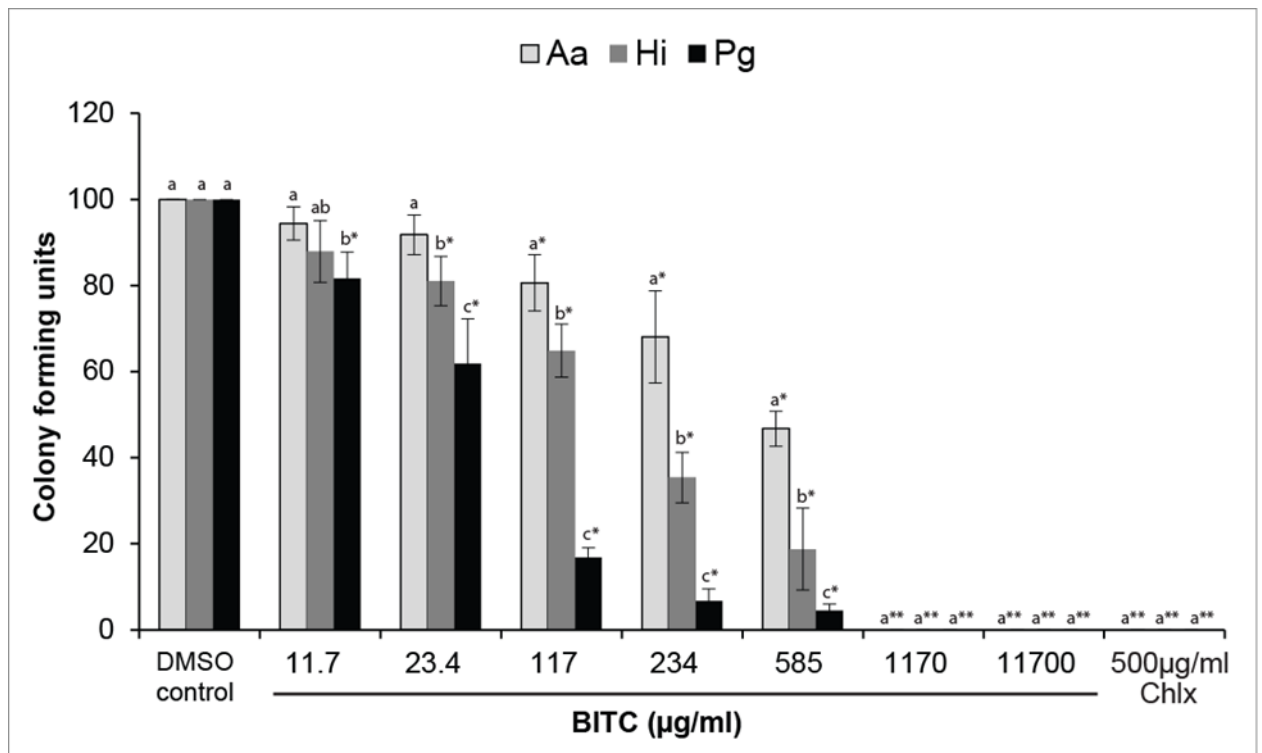


Fig. 15. Inhibition of growth of *A. actinomycetemcomitans* (Aa), *H. influenzae* (Hi) and *P. gingivalis* (Pg). The individual bars present the percentage of colony forming units (CFU) at various concentrations of benzyl isothiocyanate (BITC) or chlorhexidine (Chlx) compared to the negative control (DMSO). In the above figure * stands for $p < 0.01-0.001$ and ** stands for $p < 0.0001$ when number of CFUs were compared between negative control and different concentrations of BITC or Chlx. Different letters on the bars represent significant difference ($p < 0.05$) between the CFU of three bacteria tested at a given concentration of BITC or Chlx independently, **b** indicate a significant difference compared to **a**, **c** indicate a difference compared to both **a** and **b**.

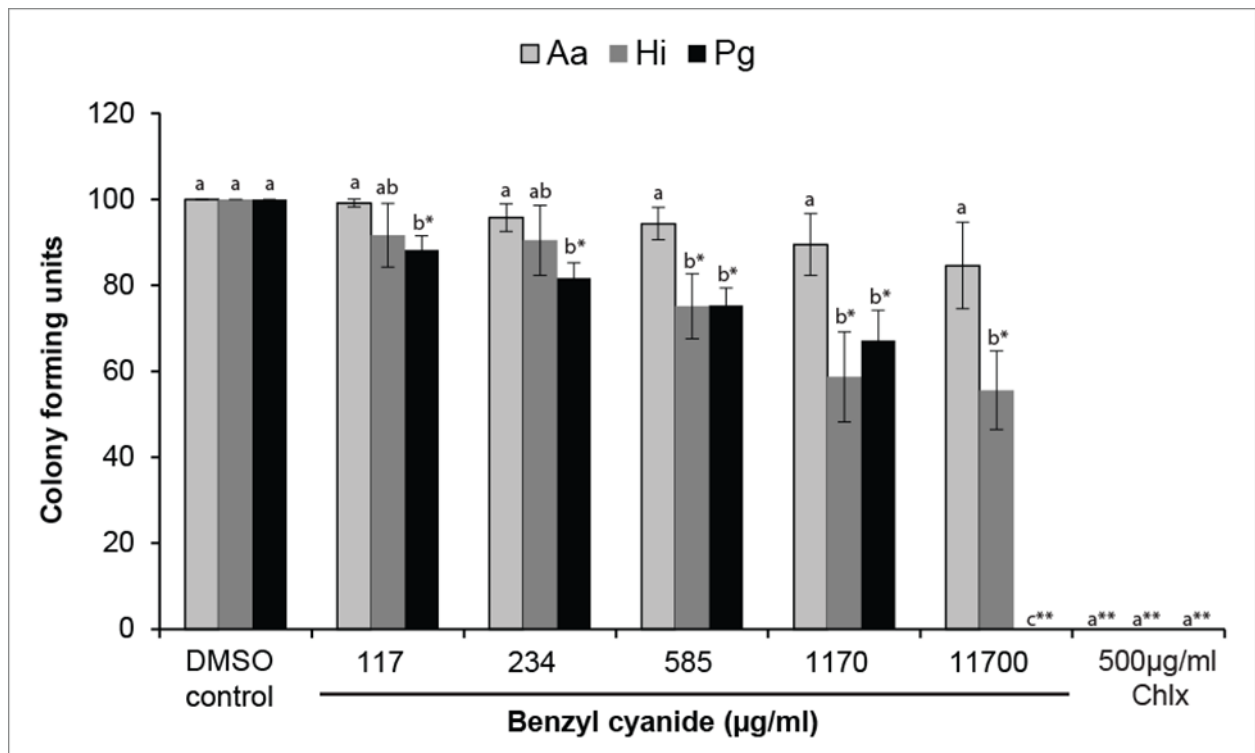


Fig.16. Inhibition of growth of *A. actinomycetemcomitans* (Aa), *H. influenzae* (Hi) and *P. gingivalis* (Pg). The individual bars present the percentage of colony forming units (CFU) at various concentrations of benzyl cyanide or chlorhexidine (Chlx) compared to the negative control (DMSO). In the above figure * stands for $p < 0.01-0.001$ and ** stands for $p < 0.0001$ when number of CFUs were compared between negative control and different concentrations of benzyl cyanide or Chlx. Different letters on the bars present significant difference ($p < 0.05$) between the CFU of three bacteria tested at a given concentration of benzyl cyanide or Chlx independently, **b** indicate a significant difference compared to **a**, **c** indicate a difference compared to both **a** and **b**.

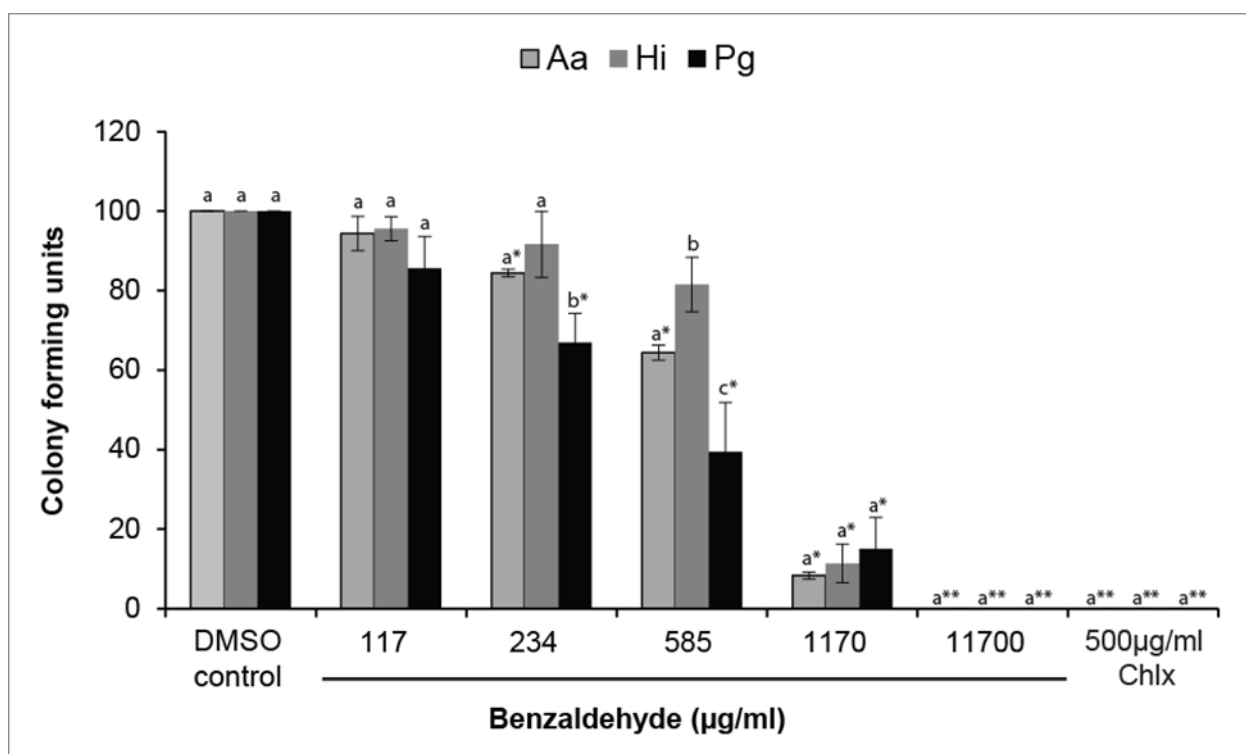


Fig. 17. Inhibition of growth of *A. actinomycetemcomitans* (Aa), *H. influenzae* (Hi) and *P. gingivalis* (Pg). The individual bars present the percentage of colony forming units (CFU) at various concentrations of benzaldehyde or chlorhexidine (Chlx) compared to the negative control (DMSO), set to 100%. In the above figure * stands for $p < 0.01-0.001$ and ** stands for $p < 0.0001$ when number of CFUs were compared between negative control and different concentrations of Miswak essential oil or Chlx. Different letters on the bars presenting significant difference ($p < 0.05$) between the CFU of three bacteria tested at a given concentration of Miswak essential oil or Chlx independently, **b** indicate a significant difference compared to **a**, **c** indicate a difference compared to both **a** and **b**

3.3 PAPER III

3.3.1 Effect of Miswak oil and BITC on the release of IL-6, IL-8 and MMP-1 by human gingival fibroblasts

We assessed the effects of Miswak oil and BITC on IL-6, IL-8, and MMP-1 secretion by non-activated and IL-1 β activated hGFs. Baseline secretion of IL-6, IL-8, and MMP-1 was detected in non-activated hGFs. IL-1 β stimulation led to a significant increase in both IL-6 and IL-8, but not MMP-1 secretion (Figure 18). Miswak oil or BITC alone had no effect on the secretion of IL-6 or IL-8 (Fig. 18A, D and Fig 18B, E). Miswak oil and BITC decreased IL-1 β induced IL-6 secretion with the strongest effects observed at 1.0 μ g/ml of Miswak oil whereas comparable effects were observed between the different concentrations of BITC (Fig 18A, D). Miswak oil and BITC decreased IL-1 β induced IL-8 secretion, with the strongest effects observed at 1.0 μ g/ml of Miswak whereas comparable effects were observed between the different concentrations of BITC (Fig. 18B, E). We also measured the levels of MMP-1 but found no significant differences neither in response to IL-1 β nor to Miswak oil or BITC (Fig 18 C, F).

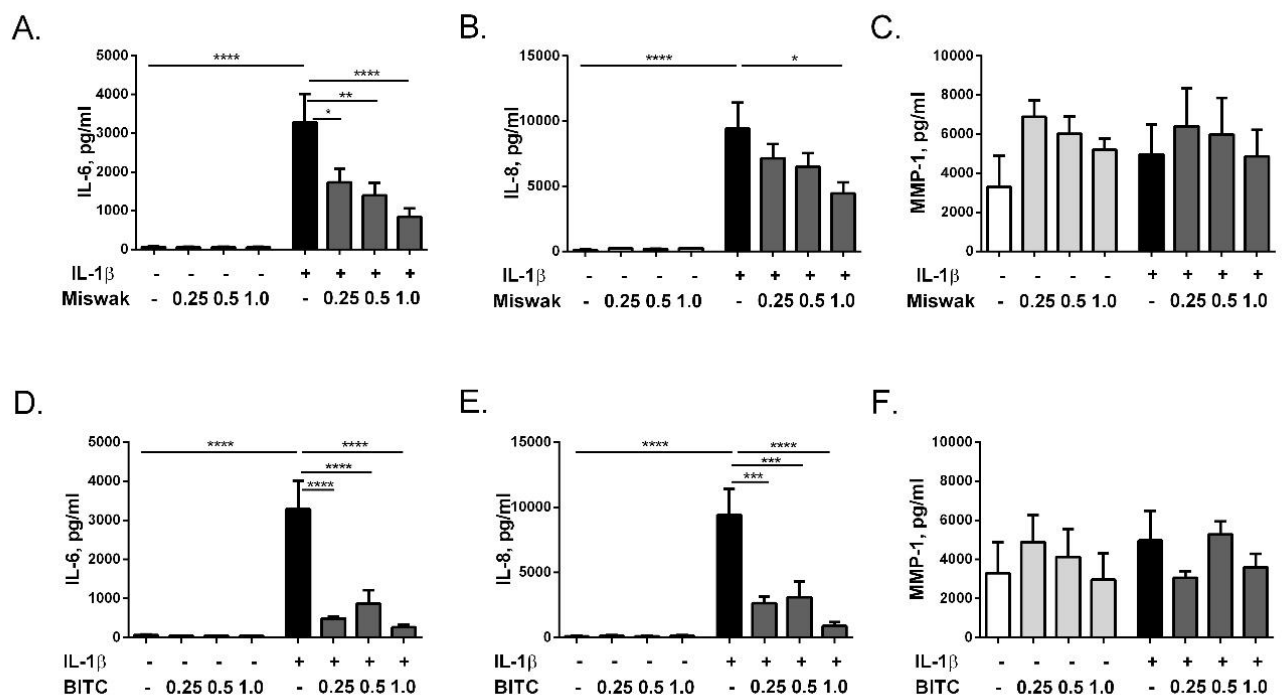


Fig.18. Effect of Miswak and BITC on IL-6, IL-8 and MMP-1 secretion from human gingival fibroblasts. The levels of IL-6, IL-8, MMP-1 secreted from non-activated or IL-1 β activated human gingival fibroblasts in the presence or absence of Miswak (A-C) or BITC (D-F) assessed in cell supernatants by ELISA. Data are presented as mean + SEM from triplicates of 2 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3.2 Effect of Miswak oil and BITC on the release of IL-6, IL-8 and MMP-1 by oral keratinocytes

We also assessed the effects of Miswak and BITC on IL-6, IL-8, and MMP-1 secretion by non-activated and IL-1 β activated oral keratinocytes. Baseline secretion of IL-6, IL-8, and MMP-1 was detected in non-activated oral keratinocytes and IL-1 β stimulation showed a significant increase in both IL-6 and IL-8, but not MMP-1 secretion (Fig. 19). Miswak oil or BITC alone had no effect on the secretion of IL-6 or IL-8 (Fig. 19A, D and Fig 19B, E). Addition of Miswak oil and BITC to IL-1 β activated keratinocytes resulted in a decrease in IL-6 which significantly observed at 0.5 and 1.0 μ g/ml of Miswak oil addition. Addition of Miswak oil to activated keratinocytes resulted in decreased IL-8 production at the concentration of 0.5 μ g/ml whereas BITC did not alter the levels of IL-8 from activated keratinocytes. We found no significant differences in MMP-1 levels neither in response to IL-1 β nor Miswak oil or BITC (Fig 19 C, F).

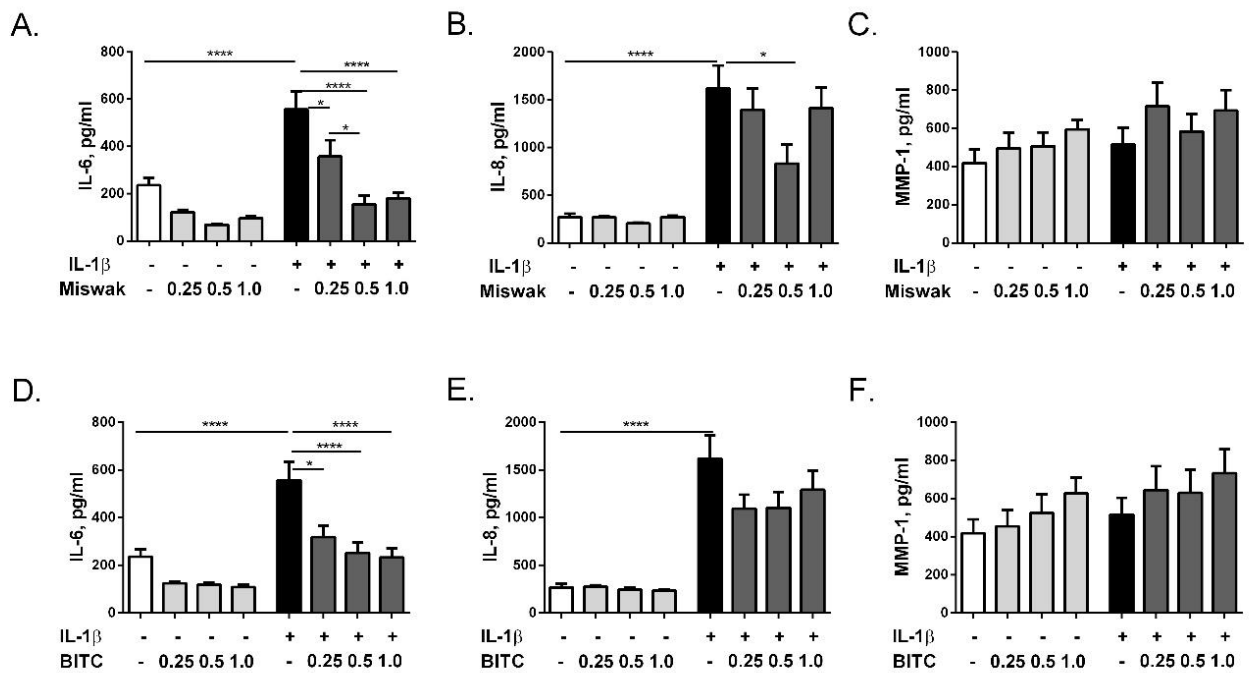


Fig 19. Effect of Miswak and BITC on IL-6, IL-8 and MMP-1 in human oral keratinocytes. The levels of IL-6, IL-8, MMP-1 from non-activated or IL-1 β activated human oral keratinocytes in the presence or absence of Miswak (A-C) or BITC (D-F) assessed in cell supernatants by ELISA. Data are presented as the Mean + SEM from triplicates of 4 experiments. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

4 DISCUSSION

The overall aim of this project was to elucidate the mechanisms for a chemical effect of chewing sticks made from *Salvadora persica* L. “Miswak”, on oral health in general and the oral microbiome in particular.

Several authors have stressed the need for proper use of the Miswak. However, at the start of this thesis, the literature had not discussed Miswak tip renewal before each use; renewing the tip of the chewing stick before use is similar to squeezing new toothpaste onto the toothbrush before brushing.

Many studies have reported a beneficial effect on oral health from brushing with Miswak. The general belief has been that the alleged effect of chewing sticks on oral health is due to mechanical cleaning alone and that the cleaning capacity of the sticks is insufficient (71) (72). In a study by Olsson (38) where he evaluated the efficiency of one type of chewing stick (Mefaka) on the oral hygiene status of Ethiopian children, he concluded that the lack of efficiency of oral cleaning with a chewing stick is likely due to faulty technique rather than to an inferior potential of the cleaning device.

Some studies found no difference in oral health between users of Miswak and users of modern toothbrushes (73, 74) while others reported higher plaque and gingivitis scores in chewing stick users. Researches explained the reduced cleaning efficiency by the longer time spent by the users chewing the stick, and that the antimicrobial substances contained in the sticks appeared to provide no additional benefits compared to the modern toothbrush and commercially available toothpastes.

While Al-Lafi and Ababneh, (75) reported in 1995 that Miswak inhibits the formation of dental plaque chemically, and exerts an antimicrobial effect against many oral bacteria, Mengel et al. (76) reported in 1996 that subjects using a toothbrush had a better periodontal status than Miswak users. Mengel et al. explained this as being due to the poor angulation of the stick during brushing and a higher frequency of using the Miswak which of course are important factors.

Thus, with full appreciation for the previous studies, at the time this thesis was being planned, the literature had not evaluated the quality of the Miswak brushing tip before and after using (once or several times) in terms of the effectiveness of chemical compounds remaining in the chewing sticks as well as released in saliva. Thus, we designed a study that would allow us to formulate guidelines for chewing stick users on how often the tip of the chewing stick should be cut in order to achieve optimal oral hygiene.

In communities where habitual Miswak use is popular, users seldom renew the tip of the stick before they brush their teeth. Rather they re-use the stick several times before cutting a new tip. The second study of this thesis found the reduced efficacy with this method. We have shown that the amount of BITC released from the Miswak decreases drastically if the same

Miswak brush is used more than once. This finding suggests that in order to achieve chemical effect, besides the mechanical, the tip of the Miswak must be renewed, that is, the old tip cut off and a new tip made by stripping off a section of bark, before each use. This finding is valuable information for the large number of habitual *S. persica* root chewing stick users.

The Sofrata et al. study found that BITC, the major active component of the fresh *S. persica* inhibits the growth of Gram negative oral bacteria (12). In the second paper of this thesis we measured the remaining amount of BITC in the used chewing sticks as well as the released amount in the saliva. Freshly cut/stripped, chewing sticks had higher quantities of BITC compared to the used chewing sticks. Compared with fresh unused chewing stick pieces, chewing stick pieces that had been used for one brushing had half the amount of BITC when the same brush was used a second time, BITC decreased further. However, BITC concentrations did not differ significantly after two times and four times use.

Fresh Miswaks released BITC into the saliva in an amount that inhibited the growth of oral pathogenic bacteria. However, we found that the released amount of BITC is retained in the saliva for a short time. This is most likely due to a combination of the volatile nature of BITC (77) and the rinsing effect of saliva. In such circumstances, increasing the frequency of Miswak use would be rationale. A previous study by Gazi et al. (46) found significantly better results when the Miswak was used five times instead of only twice in subjects with a high standard of oral hygiene.

The most commonly used substance for inhibiting plaque formation is Chlorhexidine digluconate 0.12% (78, 79). However, there is concern that this mouthwash may have deleterious effects on gingival fibroblasts and affect cell morphology, viability, and function. Other antibacterial substances that have demonstrated antibacterial activity against pathogens in the oral biofilm and have minimal side effects must be tested. Considering the strong antibacterial effect of BITC, especially on the Gram-negative bacteria associated with periodontal disease(36) , we decided to test the antibacterial effect of the average amount of salivary BITC, and other Miswak components released into the saliva, on three periodontopathic bacteria to determine whether these amounts were toxic.

Our antibacterial tests revealed that *P. gingivalis* was the most sensitive of the three tested bacteria to salivary amount of BITC. This is particularly interesting since this bacteria is considered a keystone pathogen in the etiology of periodontitis (80). The other possible antibacterial Miswak compounds that we tested, such as BA and BC, exhibited lower inhibitory effects on the tested bacteria.

MTT is an important and widely used cytotoxic assay (81, 82). The cytotoxic effects of Miswak oil and BITC were highly dependent on exposure time. For instance, after 10min of exposure to these, hGF viability was significantly reduced at BITC concentrations of 10µg/ml, which is below the average concentration detected in saliva immediately after brushing with a fresh Miswak brush. Increasing the exposure time to 24h significantly affected hGF viability at concentrations from 1.4 µg/ml.

In contrast, the oral keratinocytes proved to be a much more resistant than gingival fibroblasts, as there was no decrease in cell viability when the keratinocytes were exposed to the same concentrations as the hGFs. From a biological standpoint, the use of human oral cells derived directly from the direct tissues would be more relevant to the clinical setting, yet, cultivated hGFs in monolayer are very sensitive to cytotoxicity assays and the results cannot be compared to an *in vivo* situation in the mouth where they are surrounded by other cells in three dimensions. Furthermore, we found the retention time of BITC in saliva decreased drastically after 5 min of Miswak use. Therefore, the Miswak and its active compound (BITC) could be well tolerated *in-vivo*. Generally, the oral mucosa is more resistant to toxic substances than a cell culture because of the mucin and the keratin layer (83).

Earlier studies have reported that cultivated fibroblasts are very sensitive toward CHX, thus low concentrations of CHX ranging from 0.0025 to 0.12% can exhibit a 100% killing of cells (20, 84, 85). This is also in agreement with results in our group showing that 0.05% of CHX kills cells within seconds (21). This concentration of CHX is much lower than the recommended mouthwash concentration in the clinical practice. A previous study reported that ethanolic and hexane extract of *S. persica* were non-toxic to hGFs exposed for 24 h when using three different cytotoxic assays at 0.5 and 1 mg/ml concentration (86). This is 500 times more than the maximum concentrations used in this study. However, that could be due to different preparation methods of *S. persica* extract. Ezmerly et al. study in 1979 (87) reported that neither aqueous nor ethanolic *S. persica* Miswak extract were toxic to mice at doses of up to 1200 mg/kg. A more recent study that examined acute toxicity to albino mice recorded no fatalities in albino mice, even at doses as high as 2.04 g/kg. This indicates that the extract is well tolerated *in vivo* (88).

Oral keratinocytes proved to be a cell type that is much more resistant cell type to BITC or Miswak EO, surviving at higher concentrations and for longer exposure times. We observed no decrease in viability when the keratinocytes were exposed to higher concentrations, in contrast to hGFs.

Our group previously reported strong antibacterial effects of Miswak sticks, especially on Gram-negative bacteria *in vitro* (36), while the clinical study of Sofrata et al. showed no effect in the subgingival microbiota when tested on Saudi Arabian patients having gingivitis (89). We hypothesized two reasons for the differing results of these *in vitro* and *in vivo* studies: (i) the patients had few Gram negative bacteria since they only had gingivitis and not periodontitis and/or ii) since the participants in the clinical study was Saudi and to large extent habitual Miswak users, that they could have a micro flora already adapted to a possible chemical effect of the Miswak. Therefore, in our first study, we tested Miswak on patients who had periodontitis, to increase the possibility that they harbor Gram- negative bacteria, and who had never been exposed to Miswak. The study showed certain small differences that indicated Miswak was having a chemical effect on the oral microflora; however, it failed to show a specific effect on Gram- negative bacteria. A comparison between baseline and follow-up

showed that 16 of the 40 bacterial species being analyzed increased significantly in the second arm of the study among the participants using inactivated Miswak. Compared with the participants using active Miswak. Several reports have shown that supragingival plaque control does not significantly affect subgingival bacteria in deep periodontal pockets (90). Possibly, more frequent use of Miswak than twice a day would have had different results. In subjects with a high standard of oral hygiene, Gazi *et al.* 1990(46) reported significantly better clinical results when the *S. persica* Miswak was used five times a day compared twice a day. It might also be possible that it takes longer than the 3 weeks, as adopted in previous studies, is needed for a weak antibacterial effect to show a detectable clinical effect. The combined results of paper I and II indicate that it would be advantageous if a Miswak extract could be delivered at a higher concentration and with increased retention in the mouth to prolong the sustainability of the antimicrobial effect.

Since the traditional chewing sticks made from *S.persica* have been shown to exhibit various beneficial biological effects we further assessed the anti-inflammatory effect of *S.persica* EO and the main antibacterial compound BITC by measuring the release of IL-6, IL-8, and MMP-1 from IL-1 β - stimulated hGFs and OKs.

IL-6 is a multifunctional cytokine, that includes T-lymphocyte proliferation, B-lymphocyte differentiation, and the stimulation of immunoglobulin (Ig) secretion by B-lymphocytes (91). IL-6 is also thought to be a diagnostic marker of periodontitis (92). IL-8 acts as a focal recruitment of neutrophils to initiate and contribute to the inflammatory process (93), hence the chemotactic and activation effects of IL-8 on neutrophils in the inflamed tissues of the periodontium are more likely to promote periodontal tissue destruction (94).

The main finding in the anti-inflammatory study (paper III) was that both Miswak oil and BITC significantly reduced the release of IL-6 from both hGFs and OKs. This is line with an earlier study in carrageenan induced rat paw oedema that showed a significant reduction in circulating levels of IL-6 by crude ethanolic or ethyl acetate extracts of fresh Miswak (95), which suggests anti-inflammatory properties.

We also detected significantly reduced IL-8 secretion from hGFs treated with both Miswak oil and BITC. The effect on IL-8 release from OKs was limited and did not reach significance. Elevated levels of IL-6 and IL-8 have been observed in chronic forms of inflamed gingival tissues, and also in the GCF in patients with periodontitis (96, 97). A reduced release of pro-inflammatory cytokines, such as IL-6 and IL-8, could be a very valuable finding, since one important pathogenic mechanism in periodontitis is a hyper-inflammatory response.

One of the major MMPs produced by fibroblasts and periodontal ligament cells is MMP-1 (collagenase-1). Fibroblast type collagenase MMP-1 can be detected in the GCF of periodontitis patients (98). We detected a relatively high production of MMP-1 in both gingival fibroblasts and oral keratinocytes with or without activation with IL-1 β and with or without BITC or Miswak oil. In contrast to our results, a number of earlier studies have shown a strong

stimulatory effect of IL-1 β on the release of MMP-1 from the hGFs (99, 100). The reason for this discrepancy is unclear.

Our findings of a reduced cytokine release from the hGFs and OKs treated with BITC are in line with earlier studies of other lines. Lee et al. (2009) showed that the release of IL-1 β and IL-6 from murine macrophages were reduced after treatment with BITC.

As we demonstrated in the second paper, cytotoxicity *in vitro* showed that the hGFs and OKs differed in their responses to Miswak EO and its active component BITC (45). The oral keratinocytes were more resistant to different concentrations of Miswak oil and BITC than the hGFs. Our results show that the anti-inflammatory properties of Miswak EO *in vitro* are in line with the findings of clinical studies that have shown improved gingival health (45) and significantly reduced gingival inflammation (101) by using commercially available herbal mouth wash or toothpaste containing extracts of *S.persica*. The findings in this study also compare with earlier findings of triclosan, antibacterial agent that is common ingredient of dentifrices and mouth rinses, and has been shown to exhibit an inhibitory effect on gingival inflammation (102, 103).

Thus, paper III shows that Miswak oil and BITC have an anti-inflammatory effect on oral keratinocytes and gingival fibroblasts *in-vitro*. Further studies are required to investigate the effect of Miswak oil or its active component BITC on additional cytokines in gingival fibroblasts and oral keratinocytes challenged by IL-1 β or TNF- α .

Overall observations of this thesis strongly suggest that, for better oral hygiene results, Miswak should be cut before each use to ensure that fresh, unexposed Miswak is used. A positive chemical antibacterial effect of the active Miswak was observed on patients with periodontitis, but compared with users of the inactive Miswak, the difference was not significant; these data encourage further studies. Our anti-inflammatory investigations showed significant effects on IL-6 and IL-8 release. Since periodontitis is a hyper-inflammatory reaction to periodontal pathogens, a combination of an antibacterial and an anti-inflammatory effect could be a promising way to treat periodontitis.

5 CONCLUSION

This thesis found that:

- Brushing with a Miswak has shown indications of a weak chemical effect on the subgival microbiota of patients with mild periodontitis.
- BITC, one of the major component of the Miswak chewing stick, is released from the Miswak into the oral cavity in certain amount s, but retention time is short.
- A new Miswak tip should be cut before each use to ensure the release the highest possible of amount of BITC into the oral cavity.
- Miswak oil and BITC have an anti-inflammatory effect.

Methodological Considerations:

We have incorporated several methods and designs in the studies of this thesis, and they have several strengths, but also some limitations, which are discussed below.

Study I

Instead of an RCT design, we selected a crossover RCT design in order to (i) receive more precise results in comparison of the two patient treatment, active and inactive Miswak brushing, (ii) eliminate variation between patients, (iii) reduce the risk of error from such factors as selection bias and the clinician's preference, and (iv) insure that patient compliance was evenly distributed between groups. However, results were insignificant, which could be due to lower amounts of Gram-negative bacteria as a result of a prophylaxis procedure that preceded the trial 1 wk before. It is also possible that a weak antibacterial effect *in vivo* would require more frequent daily use to be clinically obvious. Despite these limitations, the study findings are promising, showing a positive tendency for an oral hygiene benefit of the Miswak, added an answer to the previous literature conclusions and encourage further studies, with modifications.

Study II & III

The first part of study II was an experimental methodological study done on healthy volunteers to assess the quality of Miswak chewing sticks by measuring the remaining amount of effective compound (BITC) and also the released amount of BITC in saliva after different frequencies of uses. We also measured the retention time of BITC in saliva. From this part we obtained the mean concentrations to apply in our second *in-vitro* investigation to test the antibacterial effect and cytotoxic effect on different time points. Critical selection of appropriate cell type is required for evaluation of cytotoxicity. Human gingival fibroblasts and oral keratinocytes had been frequently used in dental material toxicity because they are the dominant cell types in the gingival tissues and they are most likely cells to be exposed in the oral cavity so they imitate the intraoral condition. Yet, primary hGFs are very sensitive and considered to be bare cells compared to oral cavity environment and hence results can't be translated directly to clinical settings. In study III it was also an experimental study where we investigated the anti-inflammatory activities of *S.Persica* by assessment of the effect on the secretion of pro-inflammatory mediators from hGFs and OKs. The principles is fulfilled in both studies.

Future prospect

Antimicrobial Antiseptics, antibacterial, antifungal, antioxidant, antiviral, and analgesic agents derived from plants are of widespread interest in dental research.

The growing concern about the side effects caused by synthetic drugs and the increasing antibiotic resistance has prompted researchers to look for natural antimicrobial agents for prevention and treatment of periodontal disease.

Data from the present project have implicated Miswak extract as a potential candidate in future oral hygiene dentifrices applications in the treatment or the prevention of biofilm mediated diseases. It is suggested that more researches should be carried out to utilize BITC as the major effective component (or Miswak extract) in this regard.

The combined antibacterial, especially against Gram negative bacteria, and the anti-inflammatory effects makes extract from *S. persica* very interesting component in oral health care products such as toothpaste and mouth rinses, that fulfill these criteria.

However, there are several obstacles that have to overcome before this can be a reality. First of all the short time retention in the mouth, shown in study II and that's due to the rinsing effect of the saliva and the very high volatility of the active substance BITC. The retention must become longer, maybe in a similar way the SB12[®] retain the zinc in the mouth for a long time.

If these problems could be solved, a tooth paste or a mouth rinse with Miswak essential oil could become of a particular importance in complementing the mechanical infection control, especially in people who have problems maintaining a good oral hygiene. Thus, great excitement awaits further studies in this field.

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